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TRANSMISSION OF FOWL-POX BY MOSQUITOES: FURTHER OBSERVATIONS.

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IN a previous publication (1929) experiments were reported indicating that both *Culex* (*pipiens*) and *Stegomyia* (*Ædes ægypti*) are capable of transmitting the virus of fowl-pox from diseased to healthy chickens. It was shown further that the mosquitoes may transmit the disease at any time during the first 14 days after they have become infected, without the usual extrinsic incubation period noted in other insect-borne virus diseases.

The investigation referred to left a number of questions unsolved. It remained to determine (1) if an insect was capable of producing repeated infections; (2) the length of time which an insect once infected remained infectious; and finally (3) the localization and fate of the virus in the insect host.

The object of this paper is to report the results of experiments bearing on these questions.

Methods and Materials.

Virus.—The virus used in these experiments was the same as that employed before. Its purity and virulence were ascertained before the actual experiments were started.

Chickens.—Both white Leghorn and native chickens were used. During the early part of the work young chickens were not available, and some of those used were partly or wholly immune; subsequently, however, only young susceptible chickens were used.

Mosquitoes.—Both *Culex pipiens* and *Ædes ægypti* bred in the laboratory were used in these experiments. The methods of feeding, etc., were the same as those described in the previous paper (1929). The infected mosquitoes were kept in individual tubes at a temperature of 28°–30° C.

TABLE I.—*Transmission of Fowl-Pox by the Bite of Mosquitoes.*

Series.	Number of mosquitoes.	1st feed. Number positive transmissions. (A)	1st feed. Days after infective feed. (B)	1st feed. Incubation period (days). (C)	2nd feed. A.	2nd feed. B.	2nd feed. C.	3rd feed. A.	3rd feed. B.	3rd feed. C.	4th feed. A.	4th feed. B.	4th feed. C.	5th feed. A.	5th feed. B.	5th feed. C.	6th feed. A.	6th feed. B.	6th feed. C.
Stegomyia																			
1	2	2	2	12	...	...	..	...	...	...	...	...	...	...	...	...	...	...	...
2	4	4*	3	13	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3	4	4	3	10½	Fed on guinea-pig			4	8	10½	...	...	...	...	...	...	...	...	...
4	5	4	3	4	Fed on one chicken which became infected but individual mosquitoes not recorded	7	8½	Chicken positive, but individual mosquitoes and incubation period not recorded	8	...	3†	12	9½	1‡	14	8	1§	16	11
5	2	2	3	11½	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	2	1	2	15	Fed on one chicken which became infected but individual mosquitoes not recorded	6	8½	Chicken positive, but individual mosquitoes and incubation period not recorded	8	...	1§	11	14	0§	14	...	...	...	...
Culex																			
7	5	5	4	5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

A total of 30 mosquitoes were fed with six negative results. It is noteworthy that the incubation period after a *Culex* infection is 5 days, and with *Stegomyia* 8–11 days.

* Two of these lesions were probably secondary in character. It is of interest to note that secondary lesions make their first appearance a week or two after the primary, and as a rule in places where non-infected mosquitoes had fed. It appears that the point of the mosquito bite constitutes a sufficient injury or break in the epithelium to facilitate the localization of virus weeks after the bite.

† Only 3 fed; two were positive after 9 and 10 days respectively, and one only after 22 days, probably as above a secondary lesion.

‡ Three fed. § Two fed.

TRANSMISSION BY MOSQUITO BITE.

The various transmission experiments are summarized in Table I. It will be noted that a variety of experiments were made, each with several mosquitoes and a number of chickens. The first series was made with six insects, which were allowed to feed on the comb and wattle of a normal chicken 2 to 3 days after having fed on an infected one. In all cases except two distinct lesions appeared at the point of feeding 12 to 14 days after the mosquitoes had fed. In the second series the infected mosquitoes were allowed an intermediate feed on a guinea-pig before feeding on a healthy chicken. This intermediate feed on another host did not affect the results. Of four mosquitoes used all produced typical lesions 3 days and again 8 days after the primary feed on the infected fowl. The same insects therefore produced two consecutive infections in chickens despite the intermediate feedings on the guinea-pig. Incidentally

TABLE II.—*Localization of Virus in the Mosquito.*

Series.	Number of mosquitoes.	Date of infection.	Number of intermediate feedings.	Date of inoculation.	Material inoculated.	Result: Date positive.
1	1	23.i	4	3.ii	Head and proboscis;	...
					Body	...
2	2	23.i	6	8.ii	Head and proboscis;	...
					Body	...
3	4	23.i	6	8.ii	Head and proboscis;	14.ii
					Body	18.ii*
4	2 dead *Culex*	5.ii	None	9.ii	Head and proboscis;	15.ii
					Body	21.iii†
5	2 *Culex*	5.ii	Not recorded	18.ii	Head and proboscis;	...
					Body	...
6	2 *Stegomyia*	13.ii	1	18.ii	Head and proboscis;	27.ii
					Body	...
7	1 „	13.ii	5	4.iii	Head and proboscis;	20.iii
					Body	...
8	8 „	13.ii	5	4.iii	Head and proboscis;	... ‡
					Body	...
9	4 „	25.iv	2	3.v	Proboscis,	10.v
					Head.	...
					Abdomen	...
10	2 „	25.iv	2	3.v	Proboscis,	...
					Head,	...
					Abdomen	...
11	4 „	25.iv	2	3.v	Proboscis,	...
					Head,	...
					Abdomen	...

* Only a small vesicle which dried up on 19.ii.
† Vesicle very small in comparison with the one produced by the heads.
‡ Chicken was tested on 28.iii with ground vesicle material and found to be immune; the negative results are, therefore, ascribable to the immunity of the fowl.

the guinea-pig served to test the purity of the virus, since it showed no lesions and remained perfectly normal during a long period of observation. The third series of experiments was intended to ascertain the number of consecutive infections a mosquito can produce. It will be seen from the table that one mosquito produced six consecutive infections. Others produced three. Some failed to infect on one occasion, and subsequently produced typical infections.

These experiments confirm and extend the observations made by Kligler, Muckenfuss and Rivers (1929). They show clearly that fowl-pox can be readily transmitted from infected to normal chickens. They also demonstrate that a mosquito once infected remains so for some time, and will produce repeated infections, the feeding on another host having no effect on the transmission of the disease to healthy susceptible chickens.

LOCALIZATION OF VIRUS.

It remained to determine where the virus localized in the insect host, and whether it undergoes multiplication. For this purpose mosquitoes were fed on infected chickens, and at various intervals after the infective feed they were dissected and various portions of the insect's body inoculated on the comb or wattle of healthy chickens. The inoculations were made by rubbing the material in question on a small area of comb or wattle which had been slightly scarified by a dull scalpel prior to the inoculation. It is important to inoculate in this manner and not by injection; the early negative results were most probably due to faulty technique in inoculation.

The results are shown in Table II.

This table presents a number of interesting findings. The most frequent positive results were obtained by inoculating with the proboscis either with the head or alone. Only on two occasions were lesions obtained by inoculation of the thorax and abdomen, and in both instances the lesions were small and transitory. Of further interest is the fact that positive results were obtained with mosquitoes 16 and 19 days respectively after the infective feed.

EXPLANATION OF PLATE.

FIG. 1.—Arab chicken No. 3, infected by the bite of a *Stegomyia* 14 days after infecting feed. The vesicle appeared after 4 days; photograph 6 days later.

FIG. 2.—Leghorn No. 4. On the comb a lesion produced by the bite of a *Stegomyia* 16 days after its infection; lesions 2 days old. On wattle lesions produced by bites of *Culex* 4 days after their infecting feed. Lesions 5 and 7 days old.

FIG. 3.—Leghorn No. 8. Secondary lesion which appeared 27 days after a bite at point where a mosquito had fed without producing an infection.

FIG. 4.—Leghorn No. 2 Picture 43 days after infection. The lesions produced by bites are now a typical epithelioma; note the secondary lesions, especially the one on the right just over the eye.

FIG. 5.—Leghorn No. 7. Photograph 10 days after infection. On comb centre (*b*) the lesions resulting from the inoculations of the heads and proboscis, and at left (*a*) of bodies of *Stegomyia*. On wattle small vesicle at centre caused by bite of *Culex* infected 4 days before feeding on this chicken.

FIG. 6.—Leghorn No. 10. The dark area on the wattle (*a*) is a lesion produced by the inoculation of the head and proboscis of *Stegomyia* 43. The area where the body was inoculated (lower part of wattle, *b*) is entirely negative. The photograph was taken 16 days after the infection.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

From the experiments recorded in Table II, it appears that the virus localizes in the proboscis and undergoes no development in the body of the insect. It seemed of interest therefore to ascertain the survival of the fowl-pox virus on an inert object kept at the same temperature and humidity as the mosquitoes.

TABLE III.—*Survival and Infectivity of Virus on Inert Object (Silver Pins).*

Date of infection.	Date of inoculation.	Number of chicken.	Result: Date positive.
15.iv	15.iv	14	19.iv
	22.iv	15	27.iv
	3.v	16	8.v

The results of such an experiment are shown in Table III. A number of pins of fine silver wire were infected in as nearly as possible the same manner as the mosquitoes. The points of the pins were bent so that only 2–3 mm. would penetrate at a point near the lesion. The wires were inserted in cork stoppers of test-tubes and kept in the same incubator as the mosquitoes. The pins were tested immediately by pricking the comb or wattle of a normal chicken, and then re-tested in the same manner at intervals of a week. On all occasions positive transmission resulted with a minimum incubation period. It seems clear, therefore, that the virus will survive for at least 18 days on silver pins, and that repeated transmissions are possible, precisely as in the case of the mosquitoes.

DISCUSSION.

The experiments reported above are significant because they demonstrate a form of mechanical transmission which has received but scant attention. One is too apt to insist on an extrinsic life-cycle of a parasite as a criterion for transmission of a disease by the bite of an insect. In the case of the more sensitive parasites this may be the case, but even in such instances the possibility of direct transmission should be taken into account. Duke (1919, 1923) has called attention to the possible *rôle* of direct transmission of trypanosomiasis by tsetse flies in the epidemiology of the disease. A similar manner of transmission may ultimately be the solution of the vexed problem of the mode of transmission of Leishmania. Certainly in the case of the more resistant viruses, this rigid criterion does not necessarily apply any more than the converse assumption that the ready development of a parasite in an insect is proof of the transmission of that parasite by that insect.

However, whatever may be the case with other viruses, it is clear that the fowl-pox virus may readily be transmitted by the bite of the mosquito. What is even more important is the fact that the insect may remain infective for long periods even though the virus does not apparently undergo any development in it.

CONCLUSIONS.

Mosquitoes transmit fowl-pox from infected to healthy chickens.

The same infected insect may produce a number of consecutive infections over a period of at least 16 days.

Feeding on another animal species does not impair the infectivity of the insect.

The virus appears to be localized on the proboscis. Infection may be produced readily by the inoculation of the proboscis 16 to 19 days after the insect has become infected, but only rarely by the inoculation of any other part of its body.

The virus of fowl-pox behaves in the same manner on infected silver pins as it does on the insect's proboscis.

The possibility of direct mechanical transmission of virus as well as other diseases in which the mode of transmission is unknown should be more fully investigated.

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THE RELATION BETWEEN "GRASS DISEASE" OF HORSES AND BOTULISM.

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GRASS disease is an endemic seasonal disease of horses, which first appeared about twenty-five years ago in north-east Scotland, and has since then steadily increased in frequency. J. F. Tocher and co-workers (1923) concluded that this disease was a form of botulism. They claimed to have isolated a bacillus similar to *B. botulinus* from the organs of cases of grass disease. They stated that toxins prepared from this bacillus, when injected into horses, produced symptoms identical with those of grass disease. These claims produced considerable controversy. Gaiger concluded that the horse was comparatively insusceptible to botulism, and that "the symptoms and post-mortem appearances in horses which became intoxicated by feeding or inoculation with culture show nothing comparable with grass disease." The first of these conclusions is opposed to the results of other workers who have studied equine botulism and have found the horse quite susceptible.

Some authors have measured the lethal dose of the toxin of *B. botulinus*

when given subcutaneously to a horse in terms of the subcutaneous M.L.D for a 250 gm. guinea-pig.

Hart and Hayes found that 292 M.L.D of culture of Type A were fatal, and Wheeler found that horses were killed by 150 M.L.D. of Type B when this total quantity was given in ten doses at three-day intervals.

The evidence regarding the clinical signs and symptoms of equine botulism is incomplete because in some cases no mention is made of the type of *B. botulinus* used, in many cases unfiltered broth cultures were used, and in most cases the toxin was given by injection. If grass disease is due to a toxin the toxin is probably taken by mouth, and therefore this disease should be compared with the effects produced on horses by the oral administration of the toxin of *B. botulinus*.

The purpose of the present experiments was to determine whether oral administration of the filtered toxin of *B. botulinus* could produce in the horse signs and symptoms similar to those observed in grass disease. In particular the writer wished to determine whether prolonged administration of sublethal doses of the toxin of *B. botulinus* could produce a subacute or chronic intoxication resembling the subacute form of grass disease.

METHODS.

Schübel showed that *B. botulinus* grew best and produced a toxin of maximum potency in a meat broth containing liver. His method was adopted, and two flasks each containing 500 c.c. of a liver and meat broth were inoculated respectively with cultures of *B. botulinus* Type A (Burke No. 750) and Type B (Burke No. 751). The cultures were kindly supplied by the Lister Institute.

The cultures were incubated for seven days at 25° C. On the eighth day the culture was centrifuged for six hours, and a bacteria-free culture was obtained by filtration through Berkefeld filters. Phenol 0·5% was added, the culture was covered with sterile paraffin, and was stored at 0° C. The culture thus stored maintained its potency unaltered for six months. The M.L.D. of the filtered cultures, given subcutaneously to white mice of 20–25 gm. weight, was 0·001 c.c. of Type A culture and 0·0001 c.c. of Type B culture. The subcutaneous M.L.D. of Type B culture for a guinea-pig was 0·0001 c.c.

The filtered culture was given to eight horses, and the clinical effects and post-mortem changes were observed. The following protocols show the results of three typical experiments. In Experiment 1 the animal received a large overdose, in Experiment 3 the dose was about ten times the minimal lethal dose, and in Experiment 6 several sublethal doses were given.

Experiment 1: Clydesdale colt, 2 years, weight 831 lb.

14.12.27.—Pulse, 48 per min.; respirations, 14 per min.; temperature, 100·5° F.
5 p.m.: Given 5 c.c. Type A culture through a stomach-tube.
15.12.27.—10 a.m.: Normal. Feeding and drinking. Bowels active, urine passed.
2 p.m.: Would not drink water. Slight difficulty in feeding; oats dropped occasionally, but able to swallow. Appears slightly dull.
4 p.m.: Distinctly dull. Unable to drink. Not feeding.
5.30 p.m.: Horse lying down on its side. Unable to rise. Breathing regular, not rapid.

Unable to raise its head or sit up. Trotting movements of fore and hind legs at intervals. Easily exhausted. Tongue paralysed.

10 p.m.: Condition unaltered. No further movements of bowels or bladder.

Died during the night.

Post-mortem Examination.

Central nervous system.—Numerous small punctate hæmorrhages in brain, especially in right cerebral hemisphere, not so evident on left side No congestion of meninges. No evidence of thrombosis.

Pons, medulla, cerebellum, no abnormality.

Stomach.—No dilatation, full of partially digested fodder, greenish-yellow in colour. Mucous membrane not congested.

Small intestine.—Only small amount of fluid present. Petechial hæmorrhages seen on subperitoneal surface at one or two places. Numerous submucous hæmorrhages varying in size from pin-point to size of a sixpence, scattered along intestine. Mucous membrane not congested.

Large intestine.—Contents semi-solid, well formed. No congestion.

Bladder.—No congestion or hæmorrhages.

Kidneys.—Congestion of both kidneys, most marked in cortex. Capsule smooth, and stripped easily.

Lungs.—Slight hyperæmia.

Spleen.—Normal.

Liver.—One or two infarcts ½ in. to 1 in. long, and 1 in. to 2 in. deep. Surface smooth.

Experiment 3: Black pony gelding, weight 819 lb.

30.12.27.—12.30 p.m.: Given 0·1 c.c. Type B culture through a stomach-tube. Temperature, 100·5° F.; pulse, 45; respirations accelerated on account of struggling. Pupil moderately dilated.

31.12.27.—Pulse, 48; respirations, 12; temperature, 100° F. Animal appears normal. Feeding and drinking. Active. Bowels active, motions normal in consistence and colour.

1.1.28.—Pulse, 48; respirations, 12; temperature, 100° F. Animal still appears normal. No symptoms yet. Feeding well and able to drink. Fæces and urine passed.

2.1.28.—11 a.m.: Pulse, 46; respirations, 16; temperature, 99° F. Slight difficulty and slowness in breathing now apparent. No paralysis of tongue, but some difficulty in swallowing. Able to take oats, but not hay. Food dropped from mouth in chewing. Would not drink water. Slightly depressed and slow in moving. No salivation. Fæces passed of normal consistency and volume. Urine also passed.

3 p.m.: Condition unaltered.

6 p.m.: Animal lying down stretched out on right side and unable to rise. Tongue hanging out of mouth and definitely paralysed. Trotting movements of fore and hind legs. Pupil moderately dilated.

3.1.28.—Pulse, 60 and weak; respirations, 28; temperature, 98° F. Animal in same condition as on previous night. Trotting movements still present, but weaker. Small amount of fæces passed of normal consistency.

4.1.28.—10 a.m.: Pulse, imperceptible; respirations, 36; temperature, 103° F. Animal very weak. Trotting movements at long intervals and very feeble.

Died 12.30 p.m.

Post-mortem Examination.

Central nervous system.—Brain and meninges, no hyperæmia. No hæmorrhages.

Pons, medulla, cerebellum, no abnormal appearances.

Alimentary system.—Stomach, contents fluid, greenish colour. No dilatation. Petechial hæmorrhages scattered over surface of mucous membrane.

Intestines.—The small intestines contained a small quantity of thin watery contents. There was no congestion of the intestines and no hæmorrhages were present.

The large intestine was full of fæces of normal consistency and colour. No congestion or hæmorrhages.

Bladder.—Full, but not distended. Numerous petechial hæmorrhages present.

Lungs.—Slight degree of hypostatic congestion.

Liver, spleen and other organs showed no abnormality.

Experiment 6: Pony mare, weight 329 lb.

23.2.28.—Given 0·006 c.c. culture of Type B through a stomach-tube.

During the subsequent three days the animal appeared quite normal, but on 28.2.28 it was noted that the horse was eating more slowly, though all its food was taken. The next day this slowness in feeding was still apparent, but on 1.3.28 the animal was again feeding well and appeared normal.

6.3.28.—A second dose of 0·003 c.c. of the same toxin was given. Weight 308 lb. (loss of 21 lb.).

No symptoms developed, and on 12.3.28 a further dose of 0·003 c.c. was administered *per os*. The horse's weight on this date was 314 lb., and it had therefore gained 6 lb. since the last weighing on 6.3.28.

19.3.28.—No symptoms developed during the interval, and another dose of 0·003 c.c. toxin was given. The horse's weight was 287 lb (loss of 42 lb. since 23.2.28).

2.4.28.—No symptoms appeared. A dose of 0·03 c.c. culture of Type B was given. Weight 293 lb.

9.4.28.—No symptoms developed and the horse was feeding and drinking normally. A dose of 0·3 c.c. of the toxin was administered. Weight 270 lb.

On April 10th and 11th slight salivation was noticed and the animal was slow in feeding.

12.4.28.—Found down unable to rise. Galloping movements as described in previous cases. Died during the following night.

Experiment 6 shows the difficulty in producing a picture of chronic botulism in horses. The first five doses of toxin produced no definite change other than a fairly steady loss in weight. This loss of weight is summarized below. This experiment also shows that horses become immunized to the toxin of *B. botulinus* very slowly—a point noted by Wheeler.

Eight horses were killed with the toxin of *B. botulinus*, and the results are summarized in Table I.

These results show that the M.L.D. of Type B culture given orally to horses is between 0·01 and 0·006 c.c. This is equivalent to about 100 times the M.L.D. for a guinea-pig when the culture is given subcutaneously.

A comparison of these figures with those of Wheeler shows that this toxin is equally potent whether given by mouth or subcutaneously to horses.

Experiment 6.

Date.	Dose of toxin given (c.c.).	Weight in pounds.	Total loss in weight (pounds).
23.2.28	0·006	329	—
6.3.28	0·003	308	21
12.3.28	0·003	314	15
19.3.28	0·003	287	42
2.4.28	0·03	293	36
9.4.28	0·3	270	59
12.4.28	Died		

THE MODE OF ACTION OF THE TOXIN OF *B. BOTULINUS*.

Until recently it was generally assumed that the toxin of *B. botulinus* produced a paralysis of the central nervous system, and in particular affected the bulbar region. Many writers described post-mortem lesions of the central nervous system after death from botulism. In recent years, however, several authors have advanced the view that the toxin of *B. botulinus* acts peripherally. Edmunds and Long, and Edmunds and Kieper, working with cats and dogs

TABLE I.—*Summary of the Action of the Toxin of* B. botulinus *given Orally to Horses.*

	No. of Experiment.							
	1.	2.	3.	4.	5.	6.	7.	8.
Dose and type of toxin	5 c.c. A	1 c.c. A	0·1 c.c. B	0·08 c.c. B	(1) 0·001 c.c. B (2) 0·01 c.c. B (3) 0·05 c.c. B	(1) 0·006 c.c. B (2) 0·003 c.c. B (3) ,, (4) ,, (5) 0·03 c.c. B (6) 0·3 c.c. B	0·01 c.c. B	0·02 c.c. B.
Latent period . .	21 hrs.	72 hrs.	72 hrs.	72 hrs.	24 hrs. after 3rd dose	24 hrs. after 6th dose	3 days	6 days.
Interval between administration and death	36–48 hrs.	7 days	6 days	5 days	48 hrs. after 3rd dose	3 days after 6th dose	72 hrs.	10 days.
Effect on weight of animal	...	Lost 42 lb. in 4 days	...	...	Total loss of 72 lb.	Total loss of 59 lb.	...	...
Effect on pulse-rate .	None	None until just before death, when acceleration occurred	None	None	None	None	None	Normal until 2 days prior to death, when animal decumbent.
Bowels . . .	Normal	Slight constipation towards end	Constipation	Constipation	Normal	Normal	Normal	Constipation.
Tongue . . .	Paralysed	Paralysed	Paralysed	Paralysed	Paralysed	No definite paralysis	...	Not paralysed.
Feeding . . .	Unable to drink or swallow food	Swallowing impossible	Swallowing impossible	Swallowing impossible	Slow	Slow	...	Slow.
Time before death at which horse became unable to rise	About 12 hrs.	About 12 hrs.	40 hrs.	About 24 hrs.	Died during night; no prostration observed	About 24 hrs.	Died during night; no prostration observed	48 hrs.

found that this toxin produced a curare-like paralysis of the motor nerves of voluntary muscles, and a more or less complete paralysis of the parasympathetic. Dickson and Shevky experimented with cats, and concluded that the toxin of *B. botulinus* produced a partial peripheral paralysis of the parasympathetic nerves. They also found that in botulism fatigue could be produced with abnormal rapidity in the motor nerves to voluntary muscles. Schübel also found that the toxin of *B. botulinus* produced a curariform action. This hypothesis of the peripheral action of the toxin of *B. botulinus* was supported by the observations of Cowdry and Nicholson, who concluded that no definite lesions could be found in the brains of animals suffering from botulinus intoxication.

The statement that the toxin of *B. botulinus* can produce peripheral paralysis of the parasympathetic in some animals has an important bearing on the present investigation, because such a paralysis is one of the chief features of grass disease in horses.

The writer therefore paid especial attention to this point, but obtained no evidence for any impairment of the parasympathetic nerves in equine botulism. The pulse-rate was not accelerated except as a terminal event; moreover the bowels continued to function normally until shortly before death. The cases showed peripheral paralysis of various muscles, and particularly of the tongue, but some of the symptoms appeared to be due to a general intoxication rather than to peripheral paralysis. For example loss of weight was a well-marked symptom which appeared before there was evidence of muscular paralysis, and also was produced by doses too small to produce any other effect. The writer was unable to determine the cause of death with certainty, but the respiratory movements continued normally until shortly before death, and death did not appear to be due to respiratory paralysis.

The difference in the effects produced in horses as compared with those produced in cats and dogs may reasonably be attributed to the fact that the lethal dose of the toxin of *B. botulinus* is enormously greater in the latter case. A horse is killed by about 200 times the M.L.D. for a guinea-pig, and since the horse weighs about 1000 times as much as does the guinea-pig, therefore the horse is considerably more susceptible than the guinea-pig to this toxin. On the other hand, Edmunds and Long, who injected cultures of Type A intraperitoneally, found the M.L.D. for a guinea-pig was 0·01 c.c., whilst the M.L.D. for a cat or a dog was 2 doses of 10 c.c. Schübel's figures show that 400,000 times the M.L.D. for a guinea-pig is required to kill a cat. These figures show that the dose of toxin per unit body-weight required to kill a cat or a dog is at least 50 times as great as that sufficient to kill a horse.

The difference in the concentration of toxin present in susceptible and non-susceptible animals is so great that it is not surprising to find that the mode of action of the toxin also differs.

DISCUSSION.

The signs and symptoms of equine botulism and grass disease are summarized in Table II, and the post-mortem appearances in Table III. Grass disease can vary in its intensity from a fulminant form that kills in a

few hours to a chronic form that lasts for months. The signs and symptoms described in the tables are those of acute and subacute cases.

These tables show clearly that equine botulism and grass disease differ fundamentally both as regards the clinical signs and symptoms and also as regards their post-mortem appearances.

TABLE II.—*A Comparison of the Chief Signs and Symptoms of Equine Botulism and Grass Disease.*

	Botulism.	*Grass Disease.*
General condition	Appears normal during incubation period. One or two days before death the animal lies down and is unable to rise, but can make trotting movements with its legs	The animal is lethargic and listless, but remains standing until shortly before death.
Muscular tremors	Noticed in one case only	Generally present in muscles of shoulders and quarters.
Sweating	Rarely present	Usually present.
Pulse-rate	Normal (45–50)	Accelerated (60–120).
Temperature	Often subnormal; never raised	Never subnormal; usually 101–104° F.
Conjunctiva	Normal	Slightly jaundiced.
Tongue	Usually paralysed	Never paralysed.
Nostrils	No discharge	Frequently watery discharge.
Fæces	Constipation not marked; fæces normal in consistency	Constipation marked; fæces hard.
Course of disease	Acute and subacute only	Acute, subacute and chronic.

TABLE III.—*A Comparison of the Post-mortem Appearances in Equine Botulism and Grass Disease.*

	Botulism.	*Grass Disease.*
Stomach	Never distended	Usually distended.
Pyloric and ileo-cæcal sphincters	Normal	Constricted.
Large intestine	Contents normal in consistency	Contents frequently impacted and hard.
Liver	Generally healthy; occasionally some degree of passive congestion	Cloudy swelling usually present.

Signs which can be attributed to paralysis of the para-sympathetic system are an outstanding feature of grass disease.

Since these experiments were performed Dr. Reynolds and Dr. Slater have demonstrated lesions in the vagus nerves in twelve horses which died from

grass disease. They have kindly permitted me to refer to this work, which is not yet published. This lesion accounts for the characteristic rise of pulse-rate, and may be the cause of the equally characteristic dilatation of the stomach in grass disease.

Para-sympathetic paralysis also will account for the impaction of the colon which is invariably found in acute cases of grass disease. The colonic contents are usually of a hard, cement-like consistency, and the resultant weight of the organ may be so great that two men have difficulty in raising the rigid colon from the ground. Another constant feature of grass disease is injury to the liver, as indicated by a slight jaundice during life and cloudy swelling of the liver post-mortem.

On the other hand in equine botulism there is no evidence of para-sympathetic paralysis or liver injury. Paralysis of the tongue is one of the earliest and most characteristic signs of equine botulism, but this never occurs in grass disease.

In both equine botulism and in grass disease there is difficulty in swallowing, but this common feature is probably due to different causes in the two conditions. In botulism it is due to labioglossal paralysis, whereas in grass disease the probable cause is the dilatation of the stomach.

SUMMARY.

(1) Eight horses were poisoned by oral administration of filtered cultures of *B. botulinus*. The minimal lethal dose by the mouth of culture Type B was about 0·01 c.c. for a horse of 500 lb. (0·00005 c.c. per kilo). The hypodermic minimal lethal dose of this culture for a mouse of 28 gm. and for a guinea-pig of 250 gm. was 0·0001 c.c. (0·0036 c.c. and 0·0004 c.c. per kilo respectively).

(2) The symptoms produced by botulism in horses are quite different from those produced by grass disease, and some of the clinical symptoms which are apparently the same in the two diseases are probably due to different causes.

(3) The post-mortem findings in the two diseases are also quite different.

(4) Para-sympathetic paralysis is one of the chief features of grass disease, but this does not occur in equine botulism.

The horses used in these experiments were provided by the Animal Diseases Research Association, and the experiments on horses were conducted with the help of Mr. W. A. Pool, M.R.C.V.S. I desire to express my thanks to the Association and to Mr. Pool and Mr. Preston of the Animal Diseases Research Association for the assistance which made these experiments possible.

The toxin was prepared in the Bacteriological Department of Edinburgh University, and I desire to thank Prof. Mackie for the facilities which he provided.

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OBSERVATIONS REGARDING ALTERATIONS IN THE PERMEABILITY OF COLLODION MEMBRANES.

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THE permeability of membranes is a subject which is becoming of more and more interest and importance, not only to the physical chemist but also to the clinician. In physical chemistry this phenomenon has for a long time been the subject of research, and has been made use of for a variety of procedures. In clinical medicine it would also appear to play an important *rôle*, variations in the permeability of living membranes being closely connected with albuminuria and œdema, and also in all probability with many diseases where purpuric eruptions are a prominent symptom.

Clausen (1924), working with collodion membranes, found that their permeability was increased by treatment with blood-serum, urine, and extracts obtained from urines of patients who were suffering from nephrosis. He considered that the change produced was brought about by a substance which lowered the surface tension. The surface tension of the plasma (or serum) in nephrosis, as measured by the drop-weight method, is invariably low, being in fact lower than in any other disease with the exception of nutritional œdema. As the surface tension returns to normal the albuminuria diminishes, and the œdema in parenchymatous nephritis is correlated with the low serum protein. Bedson (1927), working with collodion membranes in connection with the ultra-filtration of viruses, also found that their permeability was increased after soaking in blood-serum. This was further confirmed by Bland (1928).

The interest of these observations, and their possible bearing on the problems of albuminuria and œdema, made a further study of the phenomenon seem of importance.

The technical difficulties of working with collodion membranes are well known, an important one being the difficulty of making membranes of a reproduceable permeability. Various methods were tried, such as making the membranes over mercury in a Petri dish, or pouring a measured quantity of collodion on to a glass plate floated on mercury, so as to ensure its even distribution. Although experiments were carried out with membranes made by these methods, it was found quite satisfactory to pour 2 or 3% collodion (alcohol 75%, ether 25%) on to a circular glass plate, and after pouring off the excess, to allow it to evaporate at room temperature for 5 minutes, and then immerse in water. Since the changes in permeability produced by the treatment of collodion membranes are of a gross nature, the membranes made in the above manner, though showing slight variations in permeability, may be considered as satisfactory for the purpose.

The particular technique adopted and the strength of the collodion used is set out in the protocols of the experiments. A series of membranes was prepared, and these were then, after thorough washing, allowed to remain soaking in water until required. At the time of the experiment the membrane was washed in a phosphate buffer solution, pH 7·6, and mounted in a Seitz filter, in a manner similar to that described by Bedson (1928). The same buffer solution was then filtered for 20 to 30 minutes, to ensure that the membrane was thoroughly washed, and to rule out mechanical leaks. In most cases Congo red was added to the buffer solution, and its absence in the filtrate was, in addition to a slow rate of filtration, proof that the membrane was intact.

The permeability of the membranes was tested by filtering guinea-pig serum diluted 1 in 100 with a phosphate buffer solution (M/50 phosphate, pH 7·6), and estimating the amount of protein in the filtrate. This was done by adding constant quantities of 25% trichloracetic acid to falling dilutions of the unfiltered serum and comparing the precipitates produced in these and in the filtered serum. The dilutions employed were 1/100, 1/1000, 1/10,000 and 1/100,000. All filtrations were carried out at a constant pressure of 120–130 mm. of mercury.

EXPERIMENTS.

The experiments were carried out with membranes treated as follows:

(i) Untreated membranes.
(ii) Membranes treated with rabbit serum.
(iii) Membranes treated with normal urine.
(iv) Membranes treated with urines containing excess of protein, and derived from cases of either nephritis or albuminuria of pregnancy.
(v) Membranes treated with extracted lipoid-free serum.
(vi) Membranes treated with a solution of extracted lipoid-free serum albumin.
(vii) Membranes treated with a solution of extracted lipoid-free serum globulin.
(viii) Membranes treated with a colloidal solution of cholesterol in water.

The preparations of extracted lipoid-free serum and solutions of extracted serum albumin and serum globulin were prepared by Dr. L. F. Hewitt by the

method which he has described (1927). The membranes that were treated were soaked in the test substance for 20 to 24 hours and were then well washed in water for 2 to 3 hours. The procedure was to compare two similar membranes, one untreated and the other treated. In some experiments, however, the same membrane was used throughout. Since Bland (1929) has shown that in the process of filtering a protein solution through a collodion membrane its permeability is gradually increased, the method of comparing the same membrane treated and untreated was abandoned, and the results obtained in such experiments were considered unreliable.

DISCUSSION.

An examination of the protocols (Tables I and II) reveals the fact that a definite increase of permeability is effected by those substances containing protein which have been the subject of investigation, the smallest changes being effected by extracted albumin and globulin. The experiments with normal urine and with cholesterol were negative. In connection with the use of Congo red for testing filters, it is of interest to mention that in many experiments where buffer solution and Congo red were filtered prior to the serum, in order to test the membrane, although no Congo red appeared in the filtrate, small quantities did so subsequent to a filtration of serum through the same membrane. This appears to be due to the fact that the Congo red becomes dispersed on the protein and thus is taken through the membrane with it.

It is difficult to say to what extent albuminuria and œdema may be due to increased permeability of the vessel walls, and it is felt that further work will be necessary before considering the possible bearing of these findings on the mechanism of these phenomena. The suggestion made by Bedson (1927) that lipoids may be the cause of this increased permeability is not confirmed by the above work, since a colloidal solution of cholesterol in water failed to increase the permeability of the membranes in those experiments in which it was employed. Since all filtrations were carried out at a constant pressure and the serum dilutions filtered were of a similar pH, neither of these factors would appear to be responsible in any way for the results obtained in different experiments.

SUMMARY AND CONCLUSIONS.

The observation that soaking collodion membranes in serum increases their permeability for serum proteins has been confirmed. The findings indicate that it is the proteins of the serum which are responsible for this phenomenon, and do not support the suggestion that the change in permeability is brought about by lipoids.

I wish to thank Dr. H. D. Kay and Dr. S. P. Bedson for their assistance and valuable advice in connection with this work, and Dr. L. F. Hewitt for kindly supplying me with the preparations of extracted serum and solutions of extracted serum albumin and globulin employed in these experiments.

TABLE I.—*2% Collodion Membranes (75% Alcohol, 25% Ether) made over Mercury. The Fractions Represent that Dilution of the Unfiltered Serum which on Addition to the Trichloracetic Acid gave an Opacity Equal to that Obtained by the Similar Treatment of the Filtrate.*

Untreated membranes.	Treated with rabbit serum.	Treated with a colloidal solution of cholesterol.
1/10,000	1/1000	1/1000
1/10,000–1/100,000	1/100–1/1000	(No opacity in filtrate).
1/100,000	1/1000	1/10,000–1/100,000
1/10,000–1/100,000	1/100–1/1000	1/100,000
1/100,000	1/1000–1/10,000	1/10,000–1/100,000
1/100,000	1/1000	
	1/1000	
	1/1000	

TABLE II.—*3% Collodion Membranes (75% Alcohol, 25% Ether) made on a Plate.*

Untreated membranes.	Treated with rabbit serum.	Treated with a solution of extracted globulin.*
1/1000	1/100–1/1000	1/1000–1/10,000
1/100,000	1/1000	1/10,000
1/10,000–1/100,000	1/1000	1/1000–1/10,000
1/10,000		1/1000–1/10,000
1/1000–1/10,000	Treated with a solution of extracted albumin.*	1/1000–1/10,000
1/100–1/1000		1/100,000
1/1000	1/100–1/1000	
1/1000	1/100,000	Treated with normal urine.
1/10,000–1/100,000	1/1000	1/100,000
1/100,000	1/1000–1/10,000	1/1000–1/10,000
1/100,000	1/100–1/1000	1/100,000
1/100,000	1/100,000	1/1000–1/10,000
1/100,000	1/10,000	1/100,000
	1/100–1/1000	1/100,000

Treated with a colloidal solution of cholesterol.	Treated with extracted serum.	Treated with urine containing an excess of protein from cases of nephritis or albuminuria of pregnancy.†
1/100,000	1/100–1/1000	1/100–1/1000
1/10,000	1/100–1/1000	1/100–1/1000
1/100,000	1/1000	1/100–1/1000
	1/1000	1/100–1/1000
		1/100
		1/100
		1/10,000
		1/10,000

* The extracted serum albumin and globulin were dissolved in saline, the concentration being 1/10th of the concentration in serum, *i.e.* albumin *circa* 0·5%, pseudo-globulin *circa* 0·25%.

† The urines were free from macroscopic blood, and only contained on microscopic examination a few red blood-cells or leucocytes.

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FURTHER OBSERVATIONS ON THE MODE OF ACTION OF A VIRICIDAL SERUM.

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IN a previous communication (1928) the author produced evidence to show that herpes virus united with its specific antibody *in vitro* suggesting that the mode of action of a viricidal serum did not differ materially from that of an antibacterial serum. Andrewes (1928) and Todd (1928) working with the viruses of vaccinia and fowl plague respectively, concluded that no interaction took place between these viruses and their antisera in the test-tube. In view of this conflict in findings it was decided to carry out further experiments with herpes virus and its specific serum. The results obtained form the subject of this communication.

TECHNICAL DATA.

In these experiments an attempt has been made to adsorb virus or antibody on particles of sufficient size to allow of their being readily thrown down and washed. In this way the two reagents in the immunity reaction could be separated from one another after varying periods of contact. Various particles have been tried—colon bacilli, boiled sago particles and collodion particles. The sago particles proved unsatisfactory, but both colon bacilli and collodion particles were found to be capable of adsorbing both virus and antibody. Although a few experiments were carried out with colon bacilli, the collodion particles were much more satisfactory and only those experiments in which they were used will be described. It should be stated, however, that the results obtained in experiments in which the colon bacilli were used were in no way in conflict with those reported.

Preparation of collodion particles.—To 1–2% alcohol-ether collodion 90% alcohol was added until the mixture became opalescent. Excess of water was

then added and rapidly mixed with the collodion. The collodion settles out in particles of varying size which can be graded to a certain extent by fractional sedimentation. For the purpose of these experiments particles were chosen which settled out in about 15 minutes, the reason for this choice being that in certain of the experiments it was desired to avoid the use of the centrifuge which hastens antigen-antibody reactions. The particles were thoroughly washed with saline before use.

Herpes antiserum.—The same serum was used throughout these experiments. It was prepared in the guinea-pig by successive intra-peritoneal inoculations of living virus (guinea-pig-skin strain). Each lot of virus used in immunizing the guinea-pig was tested for the presence of bacteria by sowing on agar and in broth. In every case no growth resulted. This serum when diluted 1 in 200 gave complete neutralization of 50 M.I.D. of herpes virus, diluted 1 in 400 only partial neutralization of this amount of virus resulted. It had been kept for 5 months in the ice-chest and had been neither heated nor filtered.

Virus.—The same strain of virus has been used throughout. This is a guinea-pig-skin strain obtained from human labial vesicles 5 months ago. It has regularly given titres of 1 in 1000 or 1 in 10,000.

EXPERIMENTAL.

Absorption of Herpes Virus by Collodion Particles.

That this can be accomplished is shown by the following experiment which is one example of many. Equal parts of washed collodion particles and virus (10% suspension of herpes vesicle epithelium in M/50 phosphate, pH 7·6, clarified by short centrifugation) were mixed and placed in the ice-chest for 20 hours. The supernatant fluid was pipetted off and kept, and the particles washed three times with such an excess of saline as to dilute any virus in the fluid remaining around the collodion particles far beyond the minimal infecting dose. The virulence of the virus before and after absorption and of the collodion particles was then tested by scarification with the following result:

Virus unabsorbed.	Virus absorbed with collodion particles.	Collodion particles.
+++	+	++

(Throughout this paper the + signs indicate degrees of "take"; − signs, no "take.")

Neutralization of Herpes Virus Adsorbed on Collodion Particles.

Collodion particles 0·5 c.c. were mixed with 2·0 c.c. herpes virus (10% pad emulsion in phosphate pH 7·6) and placed in the ice-chest overnight. The mixture was then centrifuged and the supernatant fluid pipetted off and kept for virus titration. The collodion particles were washed three times in saline =collodion-herpes particles. A small portion of the collodion-herpes particles was kept for animal inoculation and the remainder divided into two equal

portions to each of which was added 1·0 c.c. herpes-antiserum diluted 1 in 10 with phosphate buffer. The two tubes were stood at room temperature. In one the contact between the particles and antiserum was no longer than the time required for the particles to settle out, viz. 15 minutes. The supernatant serum was then pipetted off, the particles washed three times with saline and kept for virulence test. The other was allowed to stand for 2 hours when it was treated in the same way as the first. The titre of the virus before and after absorption with collodion particles and the virulence of the collodion-herpes particles before and after exposure to herpes antiserum was then ascertained by animal inoculation with the following result (Table I).

TABLE I.

Virulence of collodion-herpes particles.			Titre of virus.	
Before contact with serum.	After contact with serum.		Before absorption.	After absorption.
	10 minutes.	2 hours.		
++	—	—	1 in 1000	1 in 100

A very similar experiment in which, however, 1·0 c.c. of collodion particles was used to absorb 1·0 c.c. of virus, and in which the herpes-antiserum was used in a dilution of 1 in 20 gave the following result (Table II).

TABLE II.

Virulence of collodion-herpes particles.			Titre of virus.	
Before contact with serum.	After contact with serum.		Before absorption.	After absorption.
	10 minutes.	2 hours.		
++	—	—	1 in 1000	1 in 10

Adsorption of Herpes Antibody by Collodion Particles.

In the last two experiments it is clear that herpes virus when fixed on collodion particles is neutralized when exposed to an herpes antiserum for even as short a time as 15 minutes at room temperature, and that three washings in a volume of saline sufficient to dilute any serum remaining round about the collodion particles far beyond its neutralizing power, does not restore the virulence of the adsorbed virus. It might be argued, however, that the collodion particles themselves were capable of adsorbing the herpes antibody thus fixing virus and antibody in close proximity and so leading to neutralization of the former. That collodion particles have the power of adsorbing the antibody is shown by the following experiment. Collodion particles 1·0 c.c. were mixed with 2·0 c.c. herpes antiserum and allowed to interact for 15 minutes at room temperature, during which time the particles settled out. The supernatant serum was pipetted off and its neutralizing titre ascertained, a titration of the unabsorbed serum being carried out at the same

time. Whereas the serum before absorption with collodion particles neutralized 50 M.I.D. virus when diluted 1 in 200, after absorption it was only capable of doing so in a dilution of 1 in 20. This experiment was repeated and a similar result obtained.

Avidity for Herpes Virus of Collodion Particles Treated with Herpes Antiserum.

Since collodion particles are capable of fixing either virus or antibody, the foregoing experiments carry no proof that the virus unites with its specific antibody in the test-tube. Certain observations made in the course of these experiments suggested, however, that the particles when treated with herpes antiserum became more avid for virus. This point has been investigated and the following experiments leave no doubt that this is so. Two lots of collodion particles 1·0 c.c. in volume were mixed, one with 2·0 c.c. herpes antiserum 1 in 20 in phosphate, the other with normal guinea-pig serum similarly diluted. These were left in contact for 15 minutes at room temperature, the particles separated by centrifugalization and then washed three times with saline. To each of these lots of collodion particles was then added 1·0 c.c. herpes virus 1 in 20 and the contents of the tubes mixed thoroughly and placed in the ice-chest overnight. The two tubes were centrifugalized, the supernatants kept for virus titration and the particles washed three times and kept for virulence test. This experiment has been carried out twice and the results are given below (Table III).

TABLE III.

		Virus titration.				Virulence of particles.	
		1/20	1/100	1/1000	1/10,000	Particles treated with normal serum.	Particles treated with specific serum.
Exp. 1	Unabsorbed	++	+	±	−	++	−
	Absorbed with normal serum-treated particles	+	±	−	−		
	Absorbed with specific serum-treated particles	−	−	−	−		
Exp. 2	Unabsorbed	++	+	±	∓	++	−
	Absorbed with normal serum-treated particles	+	+	±	−		
	Absorbed with specific serum-treated particles	±	∓	−	−		

A third experiment was made to elucidate this point.

Collodion particles were sensitized with herpes antiserum and washed as usual. The following mixtures of particles and virus were then made:

A 1.	Serum-treated collodion particles	0·5 c.c.	+	1·0 c.c. virus	1 in 20.
A 2.	,,	,,	,,	0·2 c.c. + 1·0 c.c.	,, ,,
A 3.	,,	,,	,,	0·1 c.c. + 1·0 c.c.	,, ,,
B.	Untreated	,,	,,	1·0 c.c. + 1·0 c.c.	,, ,,

These were placed in the ice-chest overnight. The supernatant fluid was pipetted off from each tube and titrated for virus along with a sample of untreated virus. The results are recorded below (Table IV).

TABLE IV.

Sample.	Virus dilutions. 1/20	1/100	1/1000	1/10,000
Unabsorbed virus	++	+	±	∓
Supernatant A1	∓	?	—	—
,, A2	±	∓	—	—
,, A3	+	±	?	—
,, B	+	±	∓	—

In two cases the end-point is not clear, indefinite reactions occurring. This happens from time to time when the titration is made intradermally. In spite of these irregularities, however, it is clear from this experiment that slightly less active virus was left in the supernatant fluid after contact with 0·1 c.c. of collodian particles sensitized with specific serum than was left after contact with 1·0 c.c. untreated particles. This experiment is in support of the previous two which demonstrate in addition that active virus is not to be found on the sensitized particles either. It is, of course, open to contention that though the antibody taken up by the collodion particles is not removed by thrice washing in saline, it would nevertheless be given off when these particles were soaked for some time in phosphate buffer or an emulsion of guinea-pig skin. Should this occur one would not be dealing with an increased avidity of the sensitized particles for virus, but merely a liberation of the antibody with neutralization of the supernatant virus. This objection has been met in the following experiments. Collodion particles 1·0 c.c. in volume were sensitized with herpes antiserum as before and washed thrice in saline. They were suspended in 1·0 c.c. phosphate buffer, and after 10 minutes contact at room temperature centrifuged out and the supernatant phosphate pipetted off and kept. A further 1·0 c.c. of buffer was added to the particles, and they were placed in the ice-chest for 24 hours. They were then separated by centrifugation and the phosphate buffer pipetted off and kept. Finally these particles were mixed with 1 c.c. virus diluted 1 in 20 and placed in the ice-chest overnight. A control was put up at the same time with untreated particles. At the end of this period of contact the particles were separated from the virus, washed three times and tested for active virus. At the same time the two fractions of buffer to which the sensitized particles had been exposed were

titrated for the presence of herpes antibody. The results were as follows (Table V):

TABLE V.

Buffer fractions.	Dilutions of buffer fractions. 1/1	1/5	1/10	1/20	Virulence of particles. Sensitized.	Unsensitized.
10 minutes contact with sensitized particles	+	+	+	+	−	++
24 hours contact with sensitized particles	+	+	+	+		

Technique of antibody titration in buffer fractions.—Equal parts of falling dilutions of buffer fraction and virus diluted 1 in 20 (titre 1 in 10,000) mixed and stood at room temperature for 1 hour. Intradermal inoculation of 0·2 c.c. amounts.

In a further experiment the sensitized and washed collodion particles, 1·0 c.c. in volume, were mixed with 1·0 c.c. of a 10% suspension of normal plantar skin (guinea-pig) in phosphate buffer and placed in the ice-chest for 24 hours. They were then separated by centrifugation and the normal pad suspension pipetted off and kept for titration of antibody. Having washed the particles once in saline they were mixed with 1·0 c.c. virus 1 in 20 and placed in the ice-chest together with a control put up with untreated particles. After 24 hours contact at this temperature the particles were separated and washed three times. Their virulence was tested and the virus which had been in contact with the sensitized particles was also tested for active virus (Table VI).

TABLE VI.

	Dilutions of normal pad suspension. 1/1	1/5	1/10	1/20	Virulence of particles. Sensitized.	Unsensitized.	Virus remaining after contact with sensitized particles.
Before contact with sensitized particles	+	+	+	+	−	++	−
After 24 hours contact with sensitized particles	±	+	+	+			

Technique of antibody titration.—As in Table V except that virus titre was 1 in 1000.

These two experiments show that little or none of the antibody which has been fixed by the collodion particles is given off when these particles are left in contact with small volumes of phosphate buffer or a normal guinea-pig pad suspension.

DISCUSSION.

The work of Jones (1927, 1928) has shown that collodion particles are capable of adsorbing proteins and that when so treated the particles can be agglutinated by the appropriate precipitating serum. Arkwright (1914) demonstrated a somewhat similar phenomenon with washed bacilli. Jones

(1928) also found that though collodion particles are capable of adsorbing some of the antibody from a precipitating serum, the particles thus treated are not flocculated when placed in the presence of the protein specific for the adsorbed antibody. In the experiments recorded in this paper collodion particles have been shown capable of adsorbing either herpes virus or herpes antibody. This result was perhaps to be expected. What is of particular interest, however, is the increased avidity for virus possessed by the particles which have been sensitized with herpes antiserum. This fact clearly indicates affinity of herpes virus for the antibody contained in the specific neutralizing serum. It might of course still be argued that the antibody was liberated from the collodion particles in the presence of herpes virus and that neutralization took place in the supernatant fluid. But since the antibody is not liberated when the sensitized particles are soaked in phosphate buffer or a normal guinea-pig pad suspension, its liberation in the presence of virus would mean a specific and powerful attraction of virus for antibody. This attraction must lead to union otherwise it would be meaningless and one is therefore in a position to conclude that herpes virus and antibody do unite *in vitro*. It does not follow from the experimental evidence produced that the union is a firm one. It may well not be, but this would be obscured by the fact that the collodion particles are capable of holding either virus or antibody.

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THE DISTRIBUTION OF *B. TETANI* IN THE INTESTINES OF ANIMALS.

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IN a previous paper (1928) the results of an examination of a series of over 200 human stools for the presence of *B. tetani* were reported. As these results were at variance with those of a number of other workers it was thought advisable to examine another 100 specimens of fæces from adults. The method employed was similar to that used in the previous investigation.

A sample of the fæces was inoculated into meat medium and heated at 70° C. for 20 minutes. The tubes were rapidly cooled and incubated for 7 days. About 1 c.c. of the culture was then withdrawn and inoculated into Tulloch's exhaust medium to which a small portion of fresh kidney had been added. This was then incubated for a week under anaerobic conditions in a McIntosh and Fildes jar. A few drops of the culture from Tulloch's medium were transferred to the condensation water of an agar slope (Fildes, 1925), and incubated anaerobically. In no case was *B. tetani* isolated.

The fæces of a large number of animals were then examined in the same way, and *B. tetani* was isolated readily from a fair proportion. The results are given in Table I. It will be seen that a large number of the strains were atoxic, the test-dose being 1 c.c. of a 3–4-day culture in proteose peptone broth injected intramuscularly into the hind leg of a mouse. A few of the atoxic strains have been tested for the formation of tetanolysin, and it has been found that they produce as active lysin as do the toxic strains. The agglutination tests for typing the organisms were done with monovalent sera, using a 1-day culture of the organisms in proteose peptone broth. The reading of the agglutination tests is easy, as clumping takes place very rapidly at 50° C. and large flocculent masses are formed. It will be seen from the table that an equal number of toxic and atoxic types have been isolated, and that in both cases Type 5 is by far the most frequent. It is of interest to note the high proportion of positive results obtained from dogs and rats:

TABLE I.

Animal.	Number of specimens examined.	Number positive.	Toxic types.							Atoxic types.						
			1	2	3	4	5	6	7	1	2	3	4	5	6	7
Rabbit	22	3	...	...	...	...	1	...	...	...	...	...	...	2	...	...
Guinea-pig	6	2	...	...	...	...	...	...	...	...	...	...	...	2	...	...
Horses	53	8	...	1	1	...	3	...	...	...	...	...	...	3	...	...
Cows	21	4	...	2	2	...	...	...	...	...	...	...	...	...	...	...
Mice	19	1	...	...	...	...	...	...	...	...	...	...	...	1	...	...
Sheep	23	6	...	...	...	1	3	...	...	...	...	...	...	2	...	...
Pigs	13	1	...	...	...	...	1	...	...	...	...	...	...	...	...	...
Dogs	37	17	...	...	2	1	6	...	...	...	...	1	...	7	...	...
Cats	4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Rats	141	52	1	...	3	1	17	1	...	4	1	...	3	21	...	...
Soil	16	6	...	...	1	1	1	...	...	...	1	...	...	2	...	...
Carious teeth	18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Total	373	100	1	3	9	4	32	1	...	4	2	1	3	40	...	...

It has been stated (Buzello and Rahmel, 1924) that the fæces of birds do not contain *B. tetani*. This appeared somewhat remarkable as the organism must frequently be ingested, and an attempt was made to check the accuracy of this statement. As a preliminary experiment 34 specimens of freshly deposited hen's fæces were examined, using all possible precautions in the taking of the samples to avoid contamination with soil. Six of the samples yielded *B. tetani*, two toxic strains of Type 3, one atoxic strain of Type 4, and two toxic and one atoxic strain of Type 5 being isolated. In order to avoid

the criticism that this result might be due to external contamination, samples of fæces were taken from the large intestines of a further series of 24 fowls slaughtered for market. Two strains of *B. tetani* were isolated from this material, an atoxic Type 1 and an atoxic Type 5.

An attempt was made to determine whether *B. tetani* could be established in the intestinal canal of animals which were not carriers of the bacillus. Four white rats were taken and fed on a sterile diet: at weekly intervals over a period of 6 weeks the fæces were examined for *B. tetani* with negative results. The animals were then fed with a culture of a Type 5 strain of *B. tetani* and the fæces examined every 24 hours for 1 week, and thereafter every week for a period of 4 weeks. *B. tetani* was isolated from the stools readily at first, but after 4 days the stools became negative and remained so during the period of examination. Tenbroeck and Bauer (1923–24), experimenting with guinea-pigs, were able to establish *B. tetani* in the intestines of these animals and to show that agglutinins developed in the blood-serum. They also stated (1923) that human intestinal carriers of *B. tetani* developed antitoxin in the blood, and that a certain number of individuals, no longer carriers of the organism, still showed agglutinins in the serum. Coleman and Meyer (1926) were unable to demonstrate antitoxin in human carriers. Toen and Chang's results (1928) were in sharp contrast to those of Tenbroeck and Bauer. In an area where tetanus was rife they examined 83 unselected sera and found that only one contained antitoxin. It was subsequently found that this case had received 1500 units of antitoxin 2 days before blood was taken.

Twelve wild rats which had been shown to be carriers of *B. tetani* were selected and the blood-serum examined for antitoxin. 0·2 c.c. of serum was mixed with 2 M.L.D. of toxin for mice, the mixture incubated for 30 minutes and then injected into mice. In every instance the experimental animal died of tetanus.

DISCUSSION.

The stools of a large number of human beings have been examined for *B. tetani* with negative results. The organism appears to be present in the intestine of a large number of the lower animals, but an attempt to implant it in the intestine of white rats failed. The fæces of a certain number of hens contain this organism. This is easily understandable, as the bacillus must constantly be ingested. Antitoxin could not be demonstrated in the sera of 12 rats which were excreting the organism in the stool.

SUMMARY.

B. tetani has been isolated from the fæces of a large number of animals and also from the intestinal contents of the domestic fowl. The stools of human beings in this area have been consistently negative. The existence of a large number of atoxic strains as reported by Fildes has been confirmed. These show a similar distribution to the toxic types and produce tetanolysin.

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THE IMMEDIATE ACID CHANGE IN SHED BLOOD IN DISEASE.

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R. E. HAVARD and P. T. Kerridge (1929), using the glass electrode method (P. T. Kerridge, 1926; L. Bayliss, P. T. Kerridge and R. C. Verney, 1926), showed that the hydrogen ion concentration of blood increases a few minutes after the blood leaves the vein or artery. This change has a mean value of $-0{\cdot}05$ pH in normal human blood, and the average deviation from the mean is $\pm 0{\cdot}015$ pH. The time at which the change takes place depends on the temperature; it happens in less than six minutes after the blood is shed at 38°C., about 11 minutes after at 28°C., and 1½ to 2 hours after at 20°C. The change occurs in whole blood, in laked blood, and in centrifuged laked blood (free from stroma and leucocytes), but does not occur in plasma. It is not affected by anticoagulants, by sodium fluoride, which stops subsequent glycolysis, nor by thymol, heptyl alcohol, urethane, or potassium cyanide. No explanation was found for the change. A practical method for measuring the pH of blood as it is in the body is as follows: The blood is drawn into a syringe cooled to 0°C., and is kept on ice until required. It is then brought to room temperature and immediately transferred to a standardized glass electrode at 38°. The change of pH with time is noted for about 10 minutes. The previous cooling delays the acid change, so that a pH-time curve with a "plateau" is obtained. During the first few minutes (2–4) the blood attains the body temperature, and the pH correspondingly becomes quickly more acid. The pH is then steady for about 5 minutes, and it is this value which corresponds to the pH of the blood in the body. Subsequently the pH changes in an acid direction as already described.

* Working for the Medical Research Council.

In the present work the blood of a number of hospital patients in bed was studied. The object was to see whether a similar acid change took place in the blood of patients in different pathological conditions, particularly those associated with different hæmoglobin contents of the blood. By this method it was also hoped that some light might be thrown on the cause of the change.

Method.

Blood was drawn from an elbow vein, usually with stasis, as we were not concerned with the absolute value of the pH of blood in disease, but with the change in it on shedding. The blood was collected into an ice-cold syringe containing sufficient potassium oxalate and sodium fluoride to prevent coagulation and glycolysis, and a glass bead to facilitate mixing. The blood was immediately placed on ice, and kept there until its pH was measured at 38° by the glass electrode method, and the subsequent changes were followed for about half-an-hour.

Experiments were made on the blood of the following 23 cases:

Pernicious anæmia (2).
Hæmophilia (1).
Chronic myeloid leukæmia (1).
Chronic lymphatic leukæmia (1).
Purpura (1).
Carcinoma of the lung and myelophthisic anæmia (1).
Banti's disease after splenectomy (1).
Hodgkin's disease (2).
Anæmias secondary to: (*a*) bleeding gastric or duodenal ulcers (2).
(*b*) carcinoma of stomach (2).
(*c*) chronic nephritis (1).
(*d*) pulmonary tuberculosis (1).
(*e*) septicæmia (1).
(*f*) unknown causes (2).
Catarrhal jaundice (1).
Obstructive jaundice due to neoplasm (1).
Familial acholuric jaundice (1).
Jaundice, cause unknown (1).

Results.

No correlation was found between the size of the acid change on shedding and the hæmoglobin contents, the colour indices, or the white-cell contents of the bloods. The mean value was $-0{\cdot}045$ pH, and the average deviation from the mean $\pm 0{\cdot}017$ pH, both figures agreeing with those found for normal people within the limits of experimental error.

As already noted, the absolute pH measurements have no particular significance as the blood was often drawn with stasis. Nevertheless, it is of interest that in one case only was the pH lower than 7·22, and in one case only higher than 7·46. The mean value was 7·37, and the average deviation from it $\pm 0{\cdot}06$. This mean pH value is more alkaline than that found under

similar conditions for normal subjects, 7·32 (R. E. Havard and P. T. Kerridge, 1929), and may be due to the fact that the latter were not rested before the withdrawal of the blood.

CONCLUSION AND SUMMARY.

The examination of the blood of 23 patients in 19 pathological conditions has shown an acid change on shedding similar to that which occurs in the blood of normal subjects, and has given no indication of the cause.

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STUDIES IN IMMUNITY TO HÆMOLYTIC STREPTOCOCCI.—II. VARIATIONS IN THE ABILITY OF BROTH CULTURES TO WITHSTAND THE BACTERICIDAL POWER OF NORMAL HUMAN BLOOD.*

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In the course of studies on the immunity of patients infected with hæmolytic streptococci a method of testing the bactericidal power of the blood for those microbes was required. Todd (1927[1]) had described previously a method by which the changes in the population of living streptococci from time to time in defibrinated blood might be measured, employing for this purpose a mixing machine on which the blood and microbes are rotated during the period of incubation. It was thought that this might form the basis of such a bactericidal method, and many estimations have indeed been carried out. But it was evident from the start that considerable fluctuations were taking place, which, if they were not due to changes in the bactericidal power of the blood, must have been due to changes in the bacterial culture. Further inquiry showed, as Todd had done previously (1927[2]), that the ability of cultures of hæmolytic streptococci to withstand the bactericidal power of defibrinated blood varied with their age and provenance.

* This paper formed part of the essay which was awarded the Katherine Bishop Harman Prize of the British Medical Association in 1928.

† Working with a grant from the Medical Research Council.

It is intended to describe and provide an explanation for the phenomenon in this paper.

Scope of the present investigation.—It will be shown that recently isolated hæmolytic streptococci from hydrocele fluid broth cultures during the phase of active growth are, in general, able to withstand the bactericidal power of normal human defibrinated blood; whereas they are unable to do so when the number of living microbes is in process of decline. When the cocci from such a culture are insusceptible to the bactericidal power of the blood (during the growth phases) they resist phagocytosis by the polymorphonuclear leucocytes; and conversely, when they are susceptible (during the phase of decline), they can be easily phagocyted.

The intimate connection between resistance of streptococci to phagocytosis and the growth phase of the culture from which they are derived is studied further, and from this the conclusion is reached that resistance of virulent hæmolytic streptococci to phagocytosis waxes during the growth phase and wanes during the decline phase; while avirulent streptococci do not become resistant even during the growth phase. The effects of such changes in resistance on the shape of the population curve in blood from hour to hour are also examined.

Criterion of virulence.—In this paper the term virulent as applied to a strain of streptococci means that the microbes from a 16-hour hydrocele fluid broth culture are able to withstand the bactericidal power of normal human defibrinated blood sufficiently to be able to multiply in it after 1 to 3 hours; while a strain termed avirulent is unable to do this. Todd (1927[2]) has shown that, in general, strains which fall under the above definition of virulence, kill mice in small doses, while the microbes from avirulent cultures are unable to do so.

Source and cultivation of the streptococci employed.—Hæmolytic streptococci isolated from cases of puerperal fever were alone used in this work. All gave large halos of hæmolysis on human blood agar plates. The history of each strain is given under the protocols. Heart digest broth was used with the addition of 5% sterile hydrycele fluid.

THE EFFECT OF THE AGE OF THE CULTURE ON THE ABILITY OF HÆMOLYTIC STREPTOCOCCI TO RESIST THE BACTERICIDAL POWER OF HUMAN DEFIBRINATED BLOOD.

In the first experiment, broth cultures of different ages, of a hæmolytic streptococcus recently isolated from a case of puerperal fever were added to normal human defibrinated blood, incubated for 2 hours on the mixing machine, and the number of survivors determined by explantation.

Experiment 1.—A mother culture of the strain under investigation (H) which had been incubated in broth for 24 hours was kept in ice at 0° C. during the period of the experiment, and from it, at intervals, 50 c.mm. was inoculated into a fresh tube of 5% hydrocele fluid broth. These tubes were incubated at 37° C. until required. They were all tested together on one specimen of normal human defibrinated blood. 10 c.mm. of suitable dilutions of each culture was added to 100 c.mm. of the blood and incubated on the

mixing machine (Todd, 1927[1]) for 2 hours, after which time 50 c.mm. was explanted into blood agar. The number of cocci added in each instance was ascertained by the explantation of 5 c.mm. of the diluted culture. The results obtained are given in Table I:

TABLE I.

Time of incubation of the broth culture.	Number of streptococci present in 1 c.c. of the broth culture.*	Number of organisms implanted.	Number of survivors.	Percentage of implant surviving.
4 hours	14,240,000	712	8	1
8 ,,	320,000,000	320	23	7
12 ,,	250,000,000	125	226	181
20 ,,	330,000,000	165	150	90
24 ,,	308,000,000	154	29	19
40 ,,	136,000,000	136	14	10
120 ,,	14,400,000	1440	136	9

* These figures were calculated from the numbers given in the third column, taking into account the dilution in each case.

This was repeated with cultures of other strains which had received slightly different previous treatment.

TABLE II.—*Strain E, which had Previously Been Passaged 5 Times Through Mice.*

Number of hours' incubation in broth.	Number of streptococci in 1 c.c. of the broth culture.	Number of organisms implanted.	Number of survivors.	Percentage of implant surviving.
4 hours	2,272,000	1136	30,000	2640
9 ,,	43,800,000	219	14,528	6633
12 ,,	200,000,000	100	4,288	4288
16 ,,	672,000,000	336	4,256	1266
21 ,,	678,000,000	339	3,200	943
46 ,,	147,200,000	736	2,400	326
88 ,,	28,800,000	144	38	26
112 ,,	5,760,000	288	62	20

Tables I and II show that cocci derived either directly from the patient or after passage through mice can multiply in human blood, but only if the implants have been made from broth cultures receiving, in the one case, 12 hours,' and in the other, 4 to 46 hours' incubation. At other times the microbes are unable to increase in the blood. Table III gives the type of result obtained with a strain of attenuated virulence, while Table IV shows that when the strains have been previously subcultured on agar for a sufficient length of time the microbes altogether lose the power of multiplication in the blood. [It is perhaps unnecessary to add that both the strains in question were able, when isolated, to multiply in human blood.]

TABLE III.—*Strain B, which had been Subcultured 117 Times on Plain Agar.*

Number of hours' incubation in broth.	Number of streptococci in 1 c.c. of the broth culture.	Number of organisms implanted.	Number of survivors.	Percentage of implant surviving.
0 hours	12,000	60	3	5
6 ,,	4,200,000	21	34	161
12 ,,	96,000,000	48	7	14
16 ,,	64,000,000	32	1	3

TABLE IV.—*Strain H, which had been Rendered Completely Avirulent by Continued Subcultivation on Agar.*

Number of hours' incubation in broth.	Number of streptococci in 1 c.c. of the broth culture.	Number of organisms implanted.	Number of survivors.	Percentage of of implant surviving.
6 hours	278,400,000	1392	50	3
15 ,,	992,000,000	496	20	4
28 ,,	1,024,000,000	512	13	2
75 ,,	200,000,000	20	4	20

Further examination of the tables shows that when the microbes are able to multiply in the blood, they have been derived from cultures in the logarithmic phase or else in the resting phase immediately succeeding. When the microbes are from cultures in the later part of the phase of decline they are unable to increase in numbers in the blood. But with avirulent strains the cocci cannot multiply in blood even during the phase of logarithmic increase.

In the next section, the probable reasons for these variations in ability of the streptococci to overcome the bactericidal power of the normal blood will be investigated.

CHANGES WHICH TAKE PLACE IN CULTURES OF HÆMOLYTIC STREPTOCOCCI, RENDERING THEM SUSCEPTIBLE OR INSUSCEPTIBLE TO THE BACTERICIDAL POWER OF NORMAL HUMAN BLOOD.

The variable results which take place when streptococci are implanted into human blood, described in the foregoing series of experiments, may be due to one or a combination of three causes, assuming that the destruction of streptococci occurs in the interior of leucocytes, and that the normal serum contains no bactericidal substances for these microbes.

(1) Streptococci may be less readily agglutinated when young than when old.

(2) Young cultures may be able to resist phagocytosis.

(3) Young cultures may be able to multiply faster in the serum moiety of the blood than older cultures.

The possibility of (1) may be entirely eliminated from discussion in view of the fact that normal serum, which is exclusively employed in these experiments,

exerts no agglutinating effect on hæmolytic streptococci.* There remain, therefore, the other two possibilities, which are examined in the next two sections.

(2) *Question as to Whether the Phagocytability of Streptococci Varies during Growth in Broth, and Whether Such Variations are Responsible for the Differences in Ability to Withstand the Bactericidal Power of Normal Blood.*

Broth cultures of different ages of the same strain of hæmolytic streptococci were examined in parallel for (*a*) their capacity to survive and multiply in normal blood and (*b*) for their readiness to be phagocyted.

Experiment 2.—Cultures of a recently isolated strain, S, were obtained by the same method as described in Experiment 1. Two sets of investigations were then carried out with these cultures.

(*a*) *Growth experiment.*—As in Experiment 1. To ascertain the percentage increase or decrease of the cocci over two hours.

(*b*) *Phagocytic experiment.*—To ascertain the percentage of cocci from each culture which can be phagocyted. Detail: (1) The number of cocci present in 1 c.mm. of each broth culture was counted by a method described by Sir Almroth Wright for the counting of vaccines (Wright and Colebrook, 1921).

(2) 5 c.mm. of each broth culture was added to 50 c.mm. of the blood in a capsule which was incubated on the mixing machine for 15 minutes, when films were made with an opsonic spreader. The number of cocci in 100 polymorphonuclear leucocytes was then counted.

(3) The white cell and differential counts having been ascertained, by an appropriate calculation it was possible to find (*a*) the number of cocci added, (*b*) the total number of cocci phagocyted, and so, (*c*) the percentage of the added cocci which were phagocyted in the case of each culture.†, ‡, §, ‖

* Massive doses of streptococci of various ages and strains have been implanted into the serum of the individual whose blood was used for these experiments, and rotated on the mixing machine as is done in the bactericidal tests. These showed, on subsequent macroscopic and microscopic examination, both an entire absence of any form of clumping and also of chain-formation (Pfaundler's thread reaction).

† Phagocytosis by the polymorphonuclears is, in these experiments, alone considered. The amount of phagocytosis by the large mononuclears is never very large in comparison. And the error introduced by neglecting them is not great. Moreover they do not show quite the same variations in amount of phagocytosis with different cultures as do the polymorphonuclears.

‡ The *total* number of microbes in each of the cultures was practically the same, with the exception of the 5-hour culture, which was much thinner than the others. In this case the cocci were first spun down and resuspended in a smaller volume of the supernatant fluid to bring the numbers present up to the same as those in the other samples.

§ The arbitrary selection of 15 minutes for the period of mixing of leucocytes and microbes requires justification. It may be argued that a longer period of mixing might lead to considerably more phagocytosis of those cocci which are only phagocyted to a small extent in that time. This is not so. In the first place it has been shown (Hare, 1928) that provided the cocci do not resist phagocytosis, practically the whole of the implants of the order of magnitude used in this work are phagocyted in the course of 15 minutes. In other words, the system of mixing is wellnigh perfect, and when cocci are not phagocyted it is because they are resistant and not for want of opportunity. Also, longer periods of incubation before the films are spread may encourage intracellular digestion, with attendant difficulties in counting the number of cocci phagocyted.

‖ By this method the resistance of the whole of the cocci, both living and dead, in the culture is studied, and not solely that of the living organisms, as ought strictly to be done. But this need not invalidate the results, for over some portions of the growth-cycle the proportion of living to total organisms is very nearly the same, but the resistance to phagocytosis is very different. There is thus reason to believe that the resistance of the *living* organisms is in a state of flux; and succeeding experiments confirm this view.

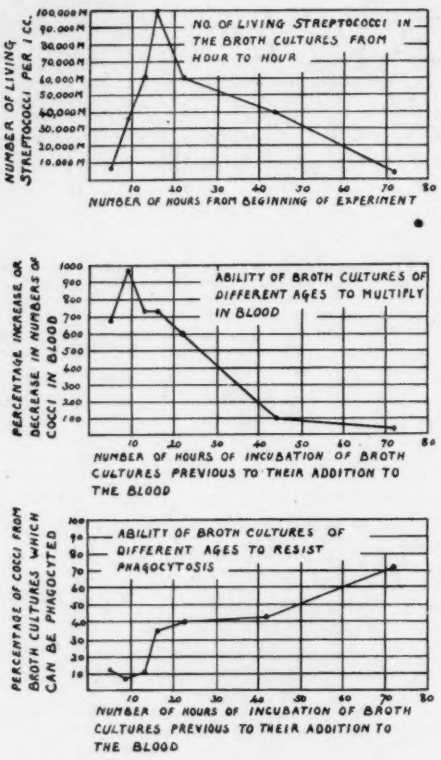
NUMBER OF LIVING STREPTOCOCCI PER 1 CC.
NO. OF LIVING STREPTOCOCCI IN THE BROTH CULTURES FROM HOUR TO HOUR
NUMBER OF HOURS FROM BEGINNING OF EXPERIMENT
PERCENTAGE INCREASE OR DECREASE IN NUMBERS OF COCCI IN BLOOD
ABILITY OF BROTH CULTURES OF DIFFERENT AGES TO MULTIPLY IN BLOOD
NUMBER OF HOURS OF INCUBATION OF BROTH CULTURES PREVIOUS TO THEIR ADDITION TO THE BLOOD
PERCENTAGE OF COCCI FROM BROTH CULTURES WHICH CAN BE PHAGOCYTED
ABILITY OF BROTH CULTURES OF DIFFERENT AGES TO RESIST PHAGOCYTOSIS
NUMBER OF HOURS OF INCUBATION OF BROTH CULTURES PREVIOUS TO THEIR ADDITION TO THE BLOOD

CHART 1. TO SHOW CONNECTION BETWEEN ABILITY TO WITHSTAND THE BACTERICIDAL POWER OF BLOOD AND THE RESISTANCE OF THE COCCI TO PHAGOCYTOSIS

The results are depicted graphically in Chart 1.

Comment.—Examination of the curve in Chart 1 indicating the amenability of the microbes to phagocytosis shows that this varies inversely as the ability to survive and multiply in normal blood. But there are evident discrepancies. For while the cocci at the 22nd hour were able to multiply freely in the blood, they were unable to do so at the 43rd hour. Yet at the former time 40% could be phagocyted and at the latter 43%. Such a small difference as this cannot account for the variations between the two cultures in ability to multiply in blood. This question is examined in a later section and a possible explanation will then be given.

Conclusions may be drawn from this: (1) That resistance to phagocytosis of hæmolytic streptococci in a broth culture is in a state of flux. (2) That these variations in resistance to phagocytosis are probably an important if not predominant factor in the varying capacity of these organisms to withstand the bactericidal elements of normal blood.

(3) *Question as to Whether the Growth-Rate of the Streptococci from Broth Cultures of Different ages are or are not always the same when they are added to Human Serum, and whether such Variations are Responsible for the Ability of Some Cultures to Survive and Multiply in Human Blood.*

Experiment 3.—Broth cultures of Strain S (recently isolated) were obtained by the same method as described in Experiment 1. The rate of growth of the microbes in normal serum during a period of 2 hours' incubation on the mixing machine was found by an exactly similar method to that described in that experiment, with the sole difference that the serum replaced the blood. The results obtained are given in Table V:

TABLE V.

Time of incubation of the broth culture.	Number of streptococci in 1 c.c. of the broth culture.	Implant.	Number of streptococci after 2 hours in the serum.	Percentage outgrowth of implanted microbes.
5 hours	89,600,000	4480	30,000	671
9 ,,	403,200,000	2016	20,000	900
13 ,,	600,000,000	300	2,400	800
16 ,,	1,000,000,000	500	3,840	768
22 ,,	600,000,000	300	864	288
44 ,,	390,400,000	1952	2,840	145

It may be concluded from this experiment that the rate of growth of hæmolytic streptococci in serum alone varies with the growth-phase of the broth culture from which they are derived, being highest with those near the logarithmic phase and lowest with those in the phase of decline. And the ability to multiply faster at certain periods than at others cannot but assist the cocci in their struggle to overcome the bactericidal power of the blood.

Consideration of the experimental results obtained in an attempted answer to the two questions discussed in this section enable the following conclusion to be drawn: Streptococci growing in broth may pass through a period of resistance to phagocytosis and ability to multiply with great rapidity in normal human serum, which coincides with part of the logarithmic phase and the early portion of the resting phase of the culture. And it is highly probable that a combination of these two attributes is, in part, responsible for the power of the cocci from broth cultures at such stages in their growth-cycle to survive the bactericidal power, and multiply to a greater or lesser degree in normal human blood; while, on the other hand, the inability of the cocci to multiply in blood when the broth culture is in the phase of decline may be due to the absence of these two attributes.

THE CHANGES IN RESISTANCE TO PHAGOCYTOSIS WHICH OCCUR AFTER THE ADDITION OF THE STREPTOCOCCI TO THE BLOOD, AND THE BEARING OF THESE CHANGES ON THE SHAPE OF THE POPULATION CURVE DURING THE SUCCEEDING PERIOD.

The changes in resistance to phagocytosis which hæmolytic streptococci undergo during culture in broth were, in the last section, invoked to explain the fluctuations in capacity to survive the bactericidal power of normal human blood displayed by these cultures. And in general, the correspondence was fairly satisfactory. But this, as an explanation, was not wholly without blemish, in that it was evidently possible to have two cultures of streptococci, both of which showed an equal degree of resistance to phagocytosis, while the one was able to multiply sixfold in normal human blood and the other was quite unable to do so (*vide* Experiment 2).

But in this experiment, and, indeed, in all of the preceding, the resistance to phagocytosis of the cultures at the moment of implantation was alone considered, while the mixtures in the bactericidal experiment were incubated for 2 hours

before explantation. In view of the fact that similar fluctuations in resistance might occur if growth took place in the serum, it was obviously of importance that the variations in resistance to phagocytosis which the cocci undergo *after* addition to the blood should also be examined.

In the next experiment the fluctuations in resistance to phagocytosis displayed by the cocci from a 24-hour broth culture of hæmolytic streptococci grown *in serum* alone at 37° C. are examined. Samples withdrawn at intervals from such a culture are diluted with fresh serum, and mixed with blood-cells to determine the amount of phagocytosis possible.

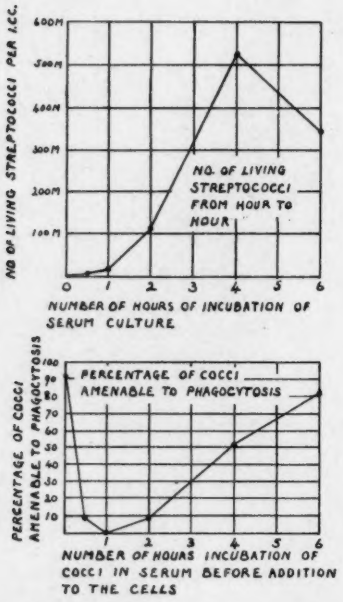
NO OF LIVING STREPTOCOCCI PER I.CC.
600M
500M
400M
300M
200M
100M
NO. OF LIVING STREPTOCOCCI FROM HOUR TO HOUR
0 1 2 3 4 5 6
NUMBER OF HOURS OF INCUBATION OF SERUM CULTURE
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
100 90 80 70 60 50 40 30 20 10
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
1 2 3 4 5 6
NUMBER OF HOURS INCUBATION OF COCCI IN SERUM BEFORE ADDITION TO THE CELLS

CHART 2. TO SHOW VARIATIONS IN RESISTANCE TO PHAGOCYTOSIS DURING GROWTH IN HUMAN SERUM

Experiment 4.—Fresh normal human serum was inoculated with hæmolytic streptococci from a 24-hour broth culture of a virulent strain (H) and incubated at 37° C. Samples were withdrawn at intervals and treated in the following manner: (1) The sample was suitably diluted with some of the uninoculated serum so that the number of microbes present after dilution would be about the same as that present in the mixture at the time of inoculation. This was, of course, omitted with the sample withdrawn at the start. 25 c.mm. of the diluted culture was then added to 25 c.mm. of mixed but unwashed blood-cells in a capsule which was then incubated on the mixing machine for 15 minutes. Films were then made with an opsonic spreader and the number of cocci in 100 leucocytes counted.

(2) The total number of microbes in the serum at each age-period was found by Sir Almroth Wright's method for the counting of vaccines.

(3) The number of white cells and the differential count of a mixture of serum and cells set up for the purpose was then found. An appropriate calculation then gave both the number of cocci added in each instance and also the number phagocyted. From these the percentage of the cocci which were phagocyted in 15 minutes was easily found.

(4) 5 c.mm. of suitable dilutions were explanted into blood agar for the determination of the number of living organisms from hour to hour. The results are shown in Table VI and Chart 2.

It is evident from this experiment that the resistance of hæmolytic streptococci does undergo profound alterations during growth in human serum. It will also be seen that the cocci which possess the greatest resistance to phagocytosis are those withdrawn from the culture near the beginning of the

logarithmic phase. (In broth the most resistant cocci are those from cultures near the summit of the logarithmic phase.)

TABLE VI.

Number of hours' incubation before addition to cells.	Total cocci present in 1 c.mm. of the mixture after dilution.	Number of cocci phagocyted by 100 polymorphs.	Total cocci phagocyted in 1 c.mm. of the mixture.	Percentage of implanted cocci phagocyted in 15 minutes.
0 hours	14,473	217	13,020	90
½ hour	13,815	21	1,060	9·1
1 „	17,105	1	60	0·3
2 hours	17,598	25	1,500	8·5
4 „	63,331	545	32,700	51
6 „	20,833	278	16,680	80

The possibility that streptococci might become highly resistant after a few minutes' growth in serum cannot but introduce complications in a bactericidal experiment, and it became essential to examine the matter in some detail. In doing so it very soon became apparent that the production of resistant cocci in serum was dependent on the age of the broth culture from which they were derived. An illustrative experiment conducted on the lines of Experiment 4 showed:

(1) Broth culture sampled near the summit of the logarithmic phase, 8–12 hours. Cocci are highly resistant to phagocytosis and continue in this condition for some hours.

(2) Broth culture sampled a little later, 12–20–30 hours. Cocci much less resistant to phagocytosis, but become much more resistant after 1–3 hours' growth in serum.

(3) Broth culture sampled when very old, 30–100 hours. Cocci almost without resistance, and do not become anything more than slightly resistant during growth in human serum.

This summary refers to the behaviour of a strain of considerable virulence, this being selected because it gives the widest series of variations. But cultures of strains of low virulence pass through a very similar cycle, the main difference being in the lower degree of severity of the resistance developed during the logarithmic phase. And attenuated strains may not become resistant at all.

In view of the results quoted in the preceding summary it seemed probable that when broth cultures of various ages were implanted into human blood the following sequence of events would occur:—

(1) Young culture: Microbes would grow out wildly from moment of implantation.

(2) Middle-aged culture: Microbes might be killed at first in large numbers but outgrowth might occur subsequently.

(3) Old culture: Most of the microbes would be killed at once and there would be no tendency to subsequent outgrowth.

In Experiment 5 these expectations are shown to be actually fulfilled.

Experiment 5.—The strain A had received five previous passages through mice. 16-, 32- and 72-hour cultures were treated alike as follows:

(*a*) The resistance of the organisms during growth in fresh human serum at 37° C. from hour to hour was measured by the same method as that described in Experiment 4.

(*b*) The rate of multiplication of the cocci in fresh normal defibrinated blood was found by incubation on the mixing machine of mixtures of 10 c.mm. of a suitable dilution of the culture and 100 c.mm. of the blood. 50 c.mm. volumes explanted at the start and at intervals into blood-agar with subsequent incubation of the plates gave the number of organisms present at each period. [The blood of the same individual was used for both parts of the experiment.]

The results are given graphically in Chart 3.

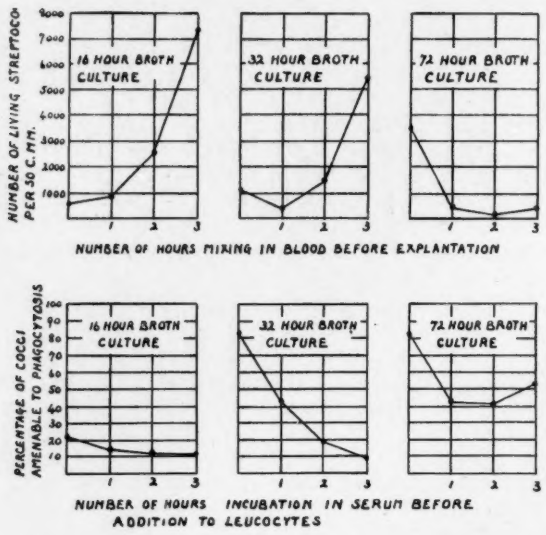
NUMBER OF LIVING STREPTOCOCCI PER 50 C.MM.
16 HOUR BROTH CULTURE
32 HOUR BROTH CULTURE
72 HOUR BROTH CULTURE
NUMBER OF HOURS MIXING IN BLOOD BEFORE EXPLANTATION
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
16 HOUR BROTH CULTURE
32 HOUR BROTH CULTURE
72 HOUR BROTH CULTURE
NUMBER OF HOURS INCUBATION IN SERUM BEFORE ADDITION TO LEUCOCYTES

CHART 3. TO SHOW THE EFFECT OF VARIATIONS IN RESISTANCE TO PHAGOCYTOSIS OCCURRING DURING GROWTH IN SERUM ON THE POPULATION CURVE OF STREPTOCOCCI IN BLOOD

Examination of the curves in Chart 3 shows that when the cocci are resistant to phagocytosis at the moment of implantation they are able to multiply in the blood from the first; but when they are not resistant they may *either* grow out eventually because the cocci become highly resistant in the serum, as occurs with the 32-hour culture, *or* they may fail to grow out, as occurs with the 72-hour culture, because this assumption of resistance fails to take place. Thus, although the 32- and 72-hour cultures were both phagocyted to the same extent at the moment of implantation, diametrically opposite results were obtained in the growth experiment.*

* The rate of growth in serum alone of the cocci from each of the three cultures was about equal. The differences in ability to withstand the bactericidal power of the blood cannot, therefore, be ascribed to this factor.

The changes which may occur in the resistance of the organisms to phagocytosis after addition to the blood are, from this experiment, as important as the changes which occur in the cultures before their addition to the blood. And it is probable that this explains the discrepancy noted in Experiment 2, where two cultures of 22 and 43 hours' growth in broth were found to be both phagocyted to the same extent (40–43%), but the former was able to multiply in blood while the latter was quite unable to do so.

APPLICATION OF THESE PRINCIPLES TO CULTURES OF HÆMOLYTIC STREPTOCOCCI IN GENERAL.

It is apparent from these experiments that streptococci pass through a series of changes in broth culture which alter them, so that they behave differently when, from time to time, they are added to human blood. While the strain employed in the last experiment was a fairly virulent strain, it is probable that most other strains pass through a very similar cycle, which has always, as its ending, utter inability to survive the bactericidal power of the blood. But the time intervals involved vary considerably with the source and previous history of each strain, some taking a long and others a very short time to become impotent. In consequence, when streptococci from a variety of sources are tested for their ability to multiply in blood after incubation in broth for 16 hours, different types of result are given according as to the rapidity or otherwise of these changes. These may be summarized as follows:

(1) *Strains of high virulence.*—No bactericidal action is evident and the cocci multiply wildly from the moment of implantation.

(2) *Strains of medium virulence.*—Bactericidal action occurs during the first hour or two hours and the numbers fall, but any survivors begin to multiply, giving a variable amount of increase in population.

(3) *Strains of low virulence.*—Bactericidal action occcurs during the first hour or two hours, but any survivors do not show a tendency to multiply.

It is therefore apparent that the population changes occurring after hæmolytic streptococci are added to human blood may be, for a variety of reasons, very variable indeed. Nevertheless it seems certain that the destruction of most strains of streptococci by normal blood must take place shortly after implantation, and certainly before the end of the second hour of incubation. For the microbes are usually in their most vulnerable condition when implanted, and any left free in the serum after the first or second hour are probably not only resistant themselves, but in process of producing more resistant cocci. This is borne out by an examination of population curves of many different strains of streptococci in several specimens of normal blood. These all show that if killing of the implanted organisms is to take place at all, the major portion of this has been effected by the end of the first hour. Thereafter, either sterilization is completed or outgrowth of a variable degree of severity occurs. Put concisely, human blood must kill streptococci quickly or not at all, for within limits, the longer they remain in it, the more chance is there of eventual outgrowth.

DISCUSSION.

Evidence has been brought forward showing that there is a definite relationship between the resistance of hæmolytic streptococci to phagocytosis and the phase of growth of the culture from which they were derived. And it is probably in consequence of this that microbes from broth cultures in different phases of growth behave differently when implanted into human blood. But besides being of considerable importance in bactericidal experiments with streptococci, the analysis of these changes is of some practical value in that it may explain the fulminating severity of infections following cuts or pricks sustained at operations on heavily infected cases. The same probably applies to puerperal or similar septic infections occuring in epidemic form. In such instances the infecting cocci are probably in a state of active division, with the almost necessary accompaniment of resistance to phagocytosis.

On the other hand, cultures which have ceased to multiply and have lost their resistance to phagocytosis may, according to their age and a number of other factors, be able to bring forth, in new medium, a new race of resistant microbes. The organisms in dried pus and discharges or in droplets from infected throats may possibly be in this condition. When such microbes are implanted into blood-clots in a wound or parturient uterus they would, probably, in the majority of cases be destroyed at once by the leucocytes in the interstices of the clots. But if this does not occur, owing possibly to some quite accidental want of contact, the microbes will grow, albeit slowly, and in so doing will digest their way towards the periphery. The process of growth is accompanied, in general, by the assumption of some degree of resistance to phagocytosis, and in consequence, when the cocci reach the serum in which the clots are bathed they are already resistant to phagocytosis. They pullulate wildly in the serum, with the result that cocci in the best possible condition for initiating an infection are disseminated into the surrounding tissues. Thereafter, some degree of local infection is almost inevitable. But all this takes time, and this will probably explain why it is that in the majority of cases of puerperal infection the first signs (temperature and pulse) occur on the third day of the puerperium.

The varying capacity of broth cultures of different ages to withstand the bactericidal power of the blood may well be the underlying cause of the results reported by Felty and Bloomfield (1924), who showed that the minimum lethal dose of hæmolytic streptococci for mice was very much smaller when the organisms were in the phase of active growth than when in the period of decline. I have performed a similar experiment using groups of 5 mice, which showed definitely that a culture of hæmolytic streptococci during the phase of active growth was more than twenty times as lethal for these animals as that in the phase of decline.

The three main types of population curve obtained with hæmolytic streptococci in normal human blood have been described. In a succeeding paper it is hoped to discuss the variations which are imposed on those curves when blood from an infected person is substituted for that of the normal. And as the serum in the majority of cases of streptococcal infection acquires opsonizing substances capable of securing the phagocytosis of resistant organisms, the

results obtained differ considerably from the curves given by the same organisms in normal blood.

CONCLUSIONS.

1. Hæmolytic streptococci from broth cultures near the logarithmic phase are better fitted to withstand the bactericidal power of normal human defibrinated blood than those from cultures in the phase of decline.

2. This is probably due, in part, to the ability of streptococci from cultures in the logarithmic phase to multiply faster in the serum moiety of the blood than those from cultures in the phase of decline, and, in part, to the assumption by growing streptococci of some degree of resistance to phagocytosis and its disappearance when the cocci become senescent.

3. The importance of this principle in experimental work on immunity and also in the epidemiology of streptococcal infections is commented upon.

I am deeply indebted to Dr. Leonard Colebrook for much assistance during the preparation of this paper.

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PRELIMINARY OBSERVATIONS ON ELECTRIC CHARGE IN ITS RELATION TO COMPLEMENT-FIXATION.

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Whilst studying the subject of electric charge in its relation to the Rieckenberg or adhesion phenomenon (Brown and Broom, 1929), it appeared that investigation of electric charge in complement-fixation reactions might throw further light upon the rationale of the process. The literature on this subject is scanty, and although there are scattered references, the subject as a whole appears never to have been investigated. Bechhold (1904) was apparently the first to demonstrate that bacteria, which carry a negative charge under normal conditions, no longer migrated in an electric field when they had been

sensitized, and he concluded that their negative charge had been reduced by the action of the serum. Northrop and De Kruif (1922), in studying the phenomenon of agglutination, made an exhaustive examination of the charge carried by bacteria under various conditions, particularly with regard to the importance of the presence and character of electrolytes. Shibley (1924) showed that immune agglutinating serum in the presence of electrolytes possessed a specific charge-reducing effect, quantitatively related to the agglutination titre of the serum. The above results were obtained with the micro-cataphoresis cell designed by Northrop (1922), and we have obtained similar results with the modification of Northrop's cell described by us (1929). Bedson and Bland (1929) introduced a simple method for studying the charges

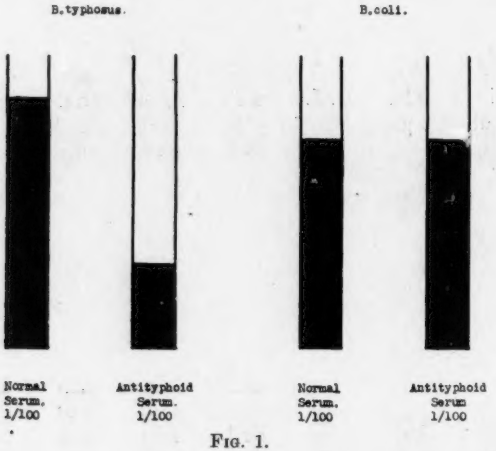

FIG. 1.

carried by particles by examining the height to which, under certain conditions, they travel up strips of filter-paper placed in contact with a solution containing them. By this also we have been able to demonstrate that the negative charge carried by bacteria is reduced by specific serum in presence of an electrolyte.

Fig. 1 shows the relative heights to which *B. typhosus* and *B. coli* travel up filter-paper in presence of inactivated normal and immune sera respectively. The bacterial suspensions in normal saline were placed in sterile watch-glasses and an equal volume of the concentration of serum added. After the fluid had risen to a height of 9 cm. the strips were removed and cut into centimetre lengths, starting from the top. These square centimetres of filter-paper were dropped into tubes of sterile broth, and after incubation at 37°C. the broth was plated out on quadrants of a MacConkey plate, and the resulting growths examined serologically.

It is thus evident that the negative charge normally carried by bacteria is considerably reduced by the action of specific immune sera in presence of an

electrolyte, and the question arises whether this alteration in charge is an important factor in the process of complement-fixation.

In the present paper we have considered complement as a whole, and have not dealt with its various fractions. As regards the charge carried by complement, we have found no reference to any work on the subject in the literature. The following experiment was therefore performed: Strips of filter-paper were suspended with the free ends immersed in fresh guinea-pig's serum diluted with normal saline. When the fluid had reached the 9-cm. mark, as before, segments of the filter-paper were teased in normal saline, and this was

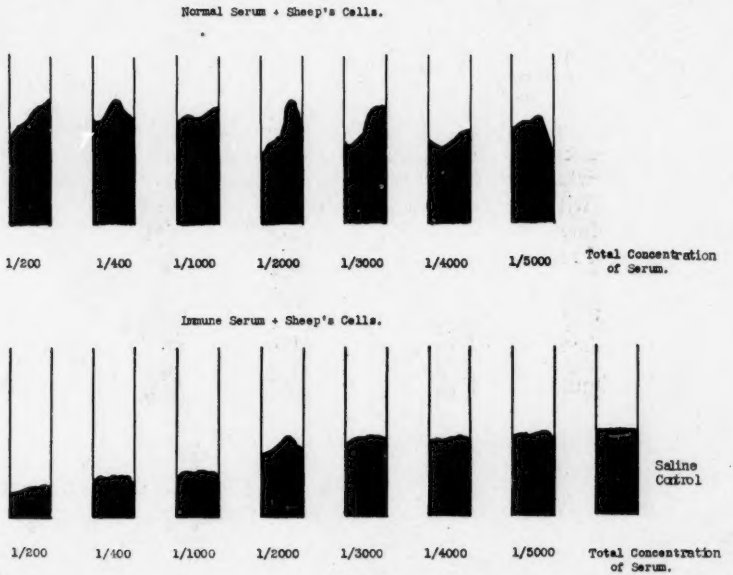

FIG. 2.

added to sensitized sheep cells. It was found that the complement had risen to the seventh centimetre segment in sufficient concentration to cause complete hæmolysis. We may conclude, therefore, that under the normal conditions of pH obtaining in complement-fixation tests, complement is negatively charged.

Sensitization of Red Cells.

It has long been known that, like bacteria, red blood-corpuscles carry a negative charge when suspended in saline, and the investigation of alterations in this charge has been undertaken by Northrop and Freund (1924), who found that the potential of cells sensitized with specific antibody was reduced to a degree dependent upon the valency of the electrolyte present.

Both by cataphoresis and by Bedson and Bland's method, we have shown that the charge of red cells is reduced in proportion to the strength of the immune serum used (Fig. 2).

Bulloch (1903) showed that sensitization of red cells is capable of taking place in hypertonic solutions, and Coulter (1921) that in absence of electrolytes the degree of sensitization depends on the pH. We also have found that electrolytes are not necessary for sensitization, and in order to investigate whether there was any alteration in the charge of the red cells sensitized in absence of an electrolyte, the following experiment was performed: Cells were washed in isotonic dextrose, and a 5% suspension was sensitized with 5 MHD of hæmolytic amboceptor, diluted in the same solution; as a control a similar suspension was prepared in normal saline. Strips of filter-paper were suspended with their free ends immersed in the suspensions, which were contained in watch-glasses, and the relative heights to which the red cells rose under the different conditions were noted when the water had risen to the 9-cm. mark. Fig. 3 shows the result of such an experiment, from which it will be seen that sensitized red cells in dextrose rise to the same height in filter-paper as unsensitized cells, while sensitized cells in saline have their charge appreciably reduced compared with normal cells in saline only.

We may therefore conclude that although no electrolyte is necessary for the sensitization of red cells, there is no reduction of charge in its absence.

Charge in its Relation to Hæmolysis.

Nolf (1900) demonstrated the inhibition of hæmolysis in electrolyte-free media and by the addition of barium salts. Bulloch (1903) found that isotonic solutions of magnesium sulphate were capable of preventing hæmolysis, although allowing sensitization to take place.

Sachs and Teruuchi (1907) showed that sensitization of red cells could take place in the absence of an electrolyte. Topley (1915) determined the optimum concentration of NaCl for the combination of complement with sensitized cells. Wright and MacCallum (1922) made an exhaustive study of the effect of electrolytes in hæmolysis. They found that the inhibition of hæmolysis in isotonic glucose solutions could be overcome by different electrolytes in varying degrees with, apparently, a relation to the valency of both ions. They formed the opinion that the order of the salts in relation to power of inhibition of hæmolysis in physiological saline, and also to that of restoring hæmolysis in glucose solution, suggests that the phenomena depend upon alterations of a physico-chemical nature in the protein constituents of the serum.

We have investigated independently the *rôle* of electrolytes in hæmolysis, using a somewhat different technique from that employed by Wright and MacCallum, and have come to the same conclusions regarding restoration of hæmolysis by salts. We are of opinion that the valency of the cation is of paramount importance provided that the salt in question has no deleterious action on the complement.

Electrolytes therefore in suitable concentrations are essential for hæmolysis, but no explanation of their mode of action has apparently been advanced.

Our contention is that the necessity for the electrolyte is that it lowers the charge of the red cells in the presence of immune serum, and that when the charge has been reduced to a critical potential, complement-fixation can occur.

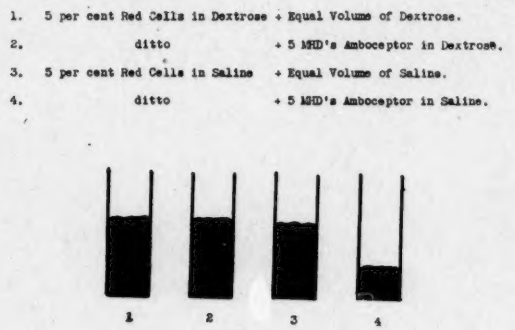
1. 5 per cent Red Cells in Dextrose + Equal Volume of Dextrose.
2. ditto + 5 MHD's Amboceptor in Dextrose.
3. 5 per cent Red Cells in Saline + Equal Volume of Saline.
4. ditto + 5 MHD's Amboceptor in Saline.
1
2
3
4

FIG. 3.

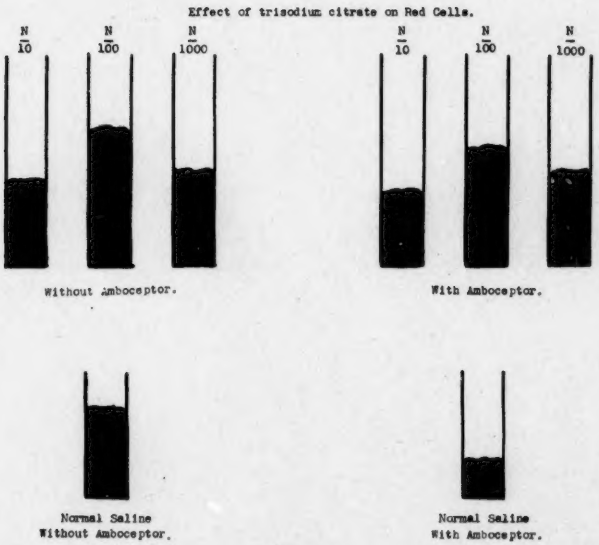

FIG. 4.

This explains the fact observed by Sachs and Teruuchi (1907), that no hæmolysis occurs when the cells are washed and the complement and amboceptor diluted in dextrose, because under these conditions there is no reduction in the charge of the cells, as we have shown in Fig. 3.

This hypothesis also offers an explanation of the effect of polyvalent anions such as trisodium citrate, which, as Gengou (1908) has shown, inhibits hæmolysis. The rationale of this inhibition appears to us to be clear when we take into consideration the work of Loeb (1924), who demonstrated that the negative charge carried by collodion particles was first increased when acted upon by salts with polyvalent anions and only reduced in high concentrations.

We have similarly found that although it is an electrolyte, the action of trisodium citrate on sensitized cells in dextrose is to maintain the negative charge of the cells (Fig. 4), and so inhibit hæmolysis.

DISCUSSION.

If charge is an important factor in complement-fixation, it might be argued that the effect of an immune serum, in presence of a suitable electrolyte, would have to be such that the sign of charge carried by the antigen was reversed before the antigen-antibody complex became attractive to complement, since complement also carries a negative charge. In the case of red cells, however, we have shown that, although their negative charge is invariably reduced before specific hæmolysis takes place, and that hæmolysis never occurs unless the charge is reduced, the sign of the charge is never reversed. There is no doubt therefore that complement-fixation does occur without a reversal of the sign of the charge carried by the antigen, and if the electrical state of the components were not an important factor in the attraction between the complement and the antigen-antibody complex, alterations in the charge of the red cells would not be of importance in the production or inhibition of hæmolysis.

We do not contend that the reduction of charge of the red cells is the sole factor of importance in complement-fixation, but that it is an essential preliminary in the process.

SUMMARY.

1. Under normal conditions the various components of complement-fixation reactions all carry negative charges.

2. The effect of electrolytes in suitable concentrations is to lower the negative charge of the antigen-antibody complex to a degree dependent upon the valency of the cation and the concentration of the immune serum.

3. Electrolytes with polyvalent anions inhibit hæmolysis because they maintain the negative charge of the sensitized cells.

4. We are of opinion that the reduction of charge of the antigen-antibody complex is an essential preliminary in the process of complement-fixation.

We wish to express our thanks to Dr. G. M. Findlay for drawing our attention to certain points in the literature on the subject.

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COMPLEMENT-FIXATION WITH FILTERABLE VIRUSES AND THEIR ANTISERA.

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GENERALLY speaking recovery from infection by a filterable virus, whether naturally or artificially induced, is associated with the appearance of specific antibody in the serum of the recovered animal. Usually the neutralization reaction is made use of to demonstrate this new activity, but a belief in the fundamental similarity in all immune responses has induced some workers to apply to viruses those test-tube reactions which occur with bacterial and other antigens and their antisera. The results obtained have been conflicting. Gordon (1925) obtained specific complement-fixation and flocculation with the viruses of vaccinia and variola, and Burgess, Craigie and Tulloch (1925) have confirmed this as far as the flocculation reaction is concerned. Ciuca (1929)

has produced evidence to show that the complement-fixation reaction can be obtained with the virus of foot and mouth disease, and Takaki, Bonis and Koref (1926) have applied this reaction in the identification of the viruses of herpes, rabies, and Japanese encephalitis. These few examples, taken from an extensive literature, show that the claims in support of the possibility of obtaining the *in vitro* immunity reactions with filterable viruses and their antisera extend to a variety of members of this group of disease agents. On the other hand, Schultz and his collaborators, in a series of communications on the subject (Schultz, 1928; Schultz, Bullock and Laurence, 1928; Schultz, Bullock and Brewer, 1928; Schultz and Hoyt, 1928), deny that specific reactions of this kind can be obtained with the filterable viruses, and attribute the reactions, when they do occur, to the presence of contaminating bacteria in the virus antigens. Their experiments are not given in detail, but one gathers from their papers that the experiments were carefully planned and thoroughly controlled. Indirect support to Schultz's contention is furnished by the work of Andrewes with vaccinia and virus III (1928) and Todd with fowl plague (1928). Both these investigators concluded that virus and antibody did not unite *in vitro*, and if this were so, of course, the necessary conditions for complement-fixation or flocculation would not occur. One of us (Bedson, 1928) has obtained evidence that specific union between herpes virus and antibody does take place outside the body, and although previous work had failed to elicit specific complement-fixation or flocculation with this virus (Bedson and Crawford, 1927), the more recent findings encouraged a re-examination of the question.

Technique of the test.—In order to avoid repetition the test may be described briefly here, and unless stated to the contrary, the following scheme may be taken as applying throughout.

(1) *Antigens.*—These consisted of suspensions in M/50 phosphate pH 7·6 of the virus-containing tissues. They were clarified by slight centrifugation or prolonged sedimentation and were unheated. They have been rarely found to be anticomplementary and have been used as a rule in a concentration of 5%. A 10% suspension in the quantity used in the test has not been found to inhibit the action of 1 M.H.D. of complement.

(2) *Antisera.*—Hyperimmune sera against the viruses of vaccinia and herpes have been prepared in the guinea-pig by repeated intraperitoneal inoculations. Some six to eight inoculations of 1·0–2·0 c.c. of a 10% virus suspension are required to produce a good serum. These sera, if comparatively recent, have been used unheated; to heat them renders them anticomplementary. When used very fresh their complement-content must be ascertained and allowance made for this in the test. After standing for some time in the cold—six months or more—they tend to become anticomplementary, but this defect can be removed by ten minutes' heating at 56°C. In the earlier work with vaccinia virus hyperimmune sera were prepared in the rabbit, but the difficulties encountered owing to the anticomplementary nature of a large percentage of rabbit sera, which furthermore cannot be got rid of in any way, led to the abandonment of this animal.

(3) *Quantities used in the test.*—0·1 c.c. serum (diluted or not as required) is mixed with 0·1 c.c. antigen and 0·1 or 0·2 c.c. complement-dilution

representing 2–4 M.H.D. Saline is added to bring the volume up to 0·5 c.c. After the necessary period of fixation 0·2 c.c. sensitized sheep cells (5% cell suspension + 5 lytic doses of amboceptor) are run in and the volumes made up to 1·0 c.c. with saline. Readings are taken after 30 minutes at 37°C. and after standing overnight in the cold.

(4) *Time and temperature of fixation.*—Indications have been obtained in earlier work (Bedson, 1928) that the union between virus and antibody takes place slowly, and these suggested that better complement-fixation might be obtained if the period of fixation were prolonged. This naturally cannot be done at 37°C. because complement deteriorates fairly rapidly at this temperature, but this does not apply to 2 to 3 hours at room temperature, or 18 to 20 hours in the ice-chest at 8 to 10°C. Experiment has shown that prolonged fixation makes all the difference between a positive and a negative or doubtful reaction, and we have adopted as a routine 2 to 3 hours' fixation at room temperature. The evidence on which this is based is given in the following section on herpes virus.

COMPLEMENT-FIXATION WITH HERPES VIRUS.

In view of Schultz's claim that positive results are due to bacterial contaminants, the principal serum used in this work—serum B 862—was prepared with virus suspensions all of which were bacterially clean. The effect of time and temperature on the results is brought out quite clearly in Tables I and II.

TABLE I.

Sera.	Time and temperature of fixation.	Antigens. Saline. 2 M.H.D.	Herpes guinea-pig pad emulsion. 2 M.H.D.	Herpes guinea-pig pad emulsion. 4 M.H.D.	Normal guinea-pig pad emulsion. 2 M.H.D.
Antiherpes B. 862 1/20	½ hr. at 37° C.	−	+	−	−
	Overnight in ice-chest approx. 10° C.	−	++++	++++	−
Saline (antigen controls)	½ hr. at 37° C.	−	−	...	−
	Overnight in ice-chest approx. 10° C.	−	−	...	−

About this time the ice-chests were replaced by refrigerators working at about 2°C., and this explains why ice-chest fixation is not represented in Table II. Fixation overnight in the refrigerator would appear superior to fixation at room temperature, and frequently it is so. But there is a tendency to non-specific fixation with the former method, as indicated by slowness of clearing in the controls, and this, together with the longer time necessary for the test, led to the adoption of fixation at room temperature for 2 to 3 hours as the routine procedure. Table III shows the result obtained when falling dilutions of antigen are put up against falling dilutions of serum.

TABLE II.

Sera.	Time and temperature of fixation.	Antigens. Saline. 2 M.H.D.	Herpes guinea-pig pad emulsion. 2 M.H.D.	Herpes guinea-pig pad emulsion. 4 M.H.D.	Normal guinea-pig pad emulsion. 2 M.H.D.
Antiherpes B. 862 1/20	½ hr. at 37° C.	—	++	—	—
		—	±	—	—
	2 hrs. at room temp.	—	++++	+++	—
		—	+++	+	—
	Overnight in refrigerator	—	++++	++++	—
		—	++++	+++	—
Saline (antigen controls)	½ hr. at 37° C.	—	—	...	—
		—	—	...	—
	2 hrs. at room temp.	—	—	...	—
		—	—	...	—
	Overnight in refrigerator	—	—	...	—
		—	—	...	—

+ + + + to ± indicate degrees of fixation. — = no fixation. The amount of complement is indicated in M H.D. at the head of each column immediately beneath the antigens. Where two readings are recorded the upper is the one made after 30 minutes at 37° C., the lower after standing overnight in the cold. This method of recording is used throughout the paper.

TABLE III.

Time and temperature of of fixation.	Anti-herpes serum B. 862.	Herpes guinea-pig pad emulsions. 1/10.	1/20.	1/30.	1/40.	1/50.	1/60.	Controls.
2 hrs. at room temperature	1/20	++++	++++	++++	+++	+	—	Antigen 1/10; —
		++++	++	+	±	—	—	2 M.H.D. comp. —
	1/40	++++	++++	+++	+	—	—	Serum B. 862 1/20; —
		+++	+	±	—	—	—	2 M.H.D. comp. —
	1/60	++++	+++	+	—	—	—	2 M.H.D. comp. —
		++	+	—	—	—	—	
	1/80	++++	++	—	—	—	—	
		+	±	—	—	—	—	

TABLE IV.

Time and temperature of fixation.	Serum.	Saline. 2 M.H.D.	Herpes guinea-pig pad emulsion. Unboiled. 2 M.H.D.	Unboiled. 4 M.H.D.	Boiled for 30 mins. 2 M.H.D.	Boiled for 30 mins. 4 M.H.D.
2 hrs. at room temperature	Antiherpes B. 862 1/20	—	++++	++++	++	—
		—	++++	++	∓	—
	Saline	—	—	...	—	...
		—	—	...	—	...

The effect of boiling the antigen for 30 minutes was next investigated in view of the work of Krans and Takaki (1926) with cocto-antigens. It is obvious from the results given in Table IV that this procedure largely reduces the efficiency of the herpes antigen.

In Table V the result of a test is given in which a vaccinia pad emulsion as well as a normal guinea-pig pad emulsion and a normal guinea-pig serum were used as controls. The fixation is seen to be quite specific.

TABLE V.

Time and temperature of fixation.	Serum.	Saline. 2 M.H.D.	Antigens. Herpes guinea-pig pad emulsion. 2 M.H.D.	Herpes guinea-pig pad emulsion. 4 M.H.D.	Vaccinia guinea-pig pad emulsion. 2 M.H.D.	Normal guinea-pig pad emulsion. 2 M.H.D.
2 hrs. at room temperature	Antiherpes B. 862 1/20	−	++++	++	−	−
		−	++++	+	−	−
	Normal guinea-pig 1/20	−	−	...	−	−
		−	−	...	−	−
	Saline	−	−	...	−	−
		−	−	...	−	−

Two old hyper-immune sera, B 369 and B 370, were then tested. These were over two years old and had not been prepared with the same care as B 862. The results are given in Table VI.

TABLE VI.

Time and temperature of fixation.	Serum.		Antigens. Herpes guinea-pig pad emulsion. 2 M.H.D.	Herpes guinea-pig pad emulsion. 4 M.H.D.	Saline. 2 M.H.D.
2 hrs. at room temperature	B. 369	Unheated	++++	++	++
			++++	+	+
		56° C. for 20 mins.	++++	−	−
			+++	−	−
	B. 370	Unheated	++++	++++	++++
			++++	+++	+++
		56° C. for 20 mins.	++	−	−
			±	−	−
	Saline		−	...	−
			−	...	−

These are good examples of sera which had become anticomplementary with age and of the removal of this defect by heating. Fixation occurs with them, but not to the same extent as with B862. It was thought that it would be of interest to compare their actual neutralizing power and to this end dilutions of 1/20, 1/100, 1/200 and 1/400 were made of each serum, and equal parts of serum dilutions and virus 1/20 (titre 1/10,000) mixed and stood at room temperature for 2 hours. They were then tested for neutralization by inoculating 0·2 c.c. of each mixture in the skin of a guinea-pig. In this experiment the proportion of virus to serum had been rather overdone, with the result that B862 1/20 was the only serum concentration which gave complete neutralization. The findings given in Table VII leave no doubt, however, that the neutralizing power places the sera in the order B862, B369 and B370, the first being the most potent, which is the order which they would assume if judged by complement-fixing power.

TABLE VII.

Serum.	Final concentration of serum in mixture.			
	1/40.	1/200.	1/400.	1/800.
B. 862	—	∓	±	+
B. 369	∓	±	+	++
B. 370	±	+	++	++

Previous work (Bedson and Crawford, 1927) had shown that the serum of a guinea-pig, convalescent from an herpetic infection, possessed definite neutralizing power for herpes virus, though only in low dilutions. The complement-fixing power of two convalescent guinea-pig sera was tested, the sera being used in a dilution of 1/2. One serum gave an entirely negative result, the other gave a trace of fixation with 2 M.H.D. of complement only. It is probably only a matter of further experimentation to make the test of sufficient delicacy to give fixation with such convalescent sera.

Altogether twelve different herpes antigens (emulsions of guinea-pig pad lesions) have been used in these experiments; all were subcultures of the same strain of virus. Two of these antigens proved anticomplementary and the reason for this was obscure. With the remaining ten antigens fixation was obtained in every case using one or other of the three sera, B862, B639 and B370. The sera were prepared with two strains of herpes virus, B862 with one, and B369 and B370 with another. Both these strains were different from the one used as antigen; unfortunately both had been lost before this work was begun. Control antigens have comprised two guinea-pig vaccinia pad emulsions and two normal guinea-pig pad emulsions. None of these was anticomplementary and none gave any fixation in the presence of any of the antiherpes sera. There seems no doubt, therefore, that the fixation obtained in these experiments is specific, and the fact that serum B862 was prepared with bacterially clean antigens disposes of Schultz's contention that the reaction is due to bacterial contaminants.

COMPLEMENT-FIXATION WITH ZOSTER VIRUS.

Netter, Urbain and Weismann-Netter (1924) have shown that the sera of convalescent zoster cases possesses the power of fixing complement in the presence of antigens composed of either zoster vesicle fluid or a suspension made from the crusts of the zoster eruption. They produced evidence that this fixation was specific, and in a number of further papers they claimed that, by means of this reaction, they had established the identity of the viruses of zoster and chicken-pox (Netter and Urbain, 1924). Their evidence has been controverted by Landa and Silberstern (1925) on the grounds that the antigens used were strongly anticomplementary and the reaction non-specific.

Some experiments, using zoster vesicle fluid as antigens and the sera from zoster convalescents, have given us such clear-cut results that we have decided to report them here, although we recognize that the number of sera and antigens worked with is small.

In Table VIII is given the result of a test in which three zoster convalescent sera and two normal sera are put up against two zoster vesicle fluids. The sera were inactivated at 56° C. and used undiluted, and although the test was only carried out against 2 M.H.D. of complement, the result leaves no doubt as to the specificity of the reaction. Antigen "S" is obviously inferior to "G," probably owing to a lower virus content; no susceptible animal being available, it is impossible to control this as in the case of herpes virus. The convalescent serum "W" is obviously weaker than "X" and "Y," which is what one would expect, since it was collected at a date further removed from the onset of disease.

TABLE VIII.

Time and temperature of fixation.	Serum.	Antigens. Zoster V.F. "G," 1/10. 2 M.H.D.	Zoster V.F. "S," 1/10. 2 M.H.D.	Saline. 2 M.H.D.
2 hrs. at room temperature	Zoster convalescent "W," 2 months	++++	∓	−
		++++	−	−
	Zoster convalescent "X," 3 weeks	++++	+++	−
		++++	++	−
	Zoster convalescent "Y," 6 weeks	++++	+++	−
		++++	++	−
	Normal	−	−	−
		−	−	−
	"	−	−	−
		−	−	−
	Saline	−	−	−
		−	−	−

The experiment recorded in Table IX confirms the findings in Table VIII, for sera "X" and "Y" are found to give fixation with a third zoster vesicle fluid, whereas two normal sera fail to do so. There is, however, another feature of this experiment which is of interest. Being in possession of a specimen of cerebro-spinal fluid from a case of encephalitis occuring in the course of a zoster infection, an attempt was made to see whether zoster virus could be demonstrated in this specimen by means of the complement-fixation reaction. As a control the cerebro-spinal fluid from a case of encephalitis complicating diphtheria was used. Both fluids were used in a dose of 0·3 c.c. in the test. The two zoster convalescent sera, "X" and "Y," both gave fixation—weakly positive it is true—with the zoster encephalitis C.S.F. but no fixation with the other specimen. The two normal sera failed to give fixation with either. This finding appears to us significant, and suggests the possibility of using the test in the elucidation of the ætiology of encephalo-myelitis.

TABLE IX.

Time and temperature of fixation.	Serum.	Antigens. Zoster V.F. "H," 1/10. 2 M.H.D.	C.S.F. zoster encephalitis, 1/1. 2 M.H.D.	C.S.F. control encephalitis, 1/1. 2 M.H.D.	Saline. 2 M.H.D.
2 hrs. at room temperature	Zoster convalescent "X"	++++	++	—	—
		+++	+	—	—
	Zoster convalescent "Y"	++++	++	—	—
		+++	±	—	—
	Normal 3	—	—	—	—
		—	—	—	—
	„ 4	—	—	—	—
		—	—	—	—
	Saline	—	—	—	—
		—	—	—	—

The specificity of the fixation obtained with zoster convalescent serum is testified to by the results recorded in Table X.

It should be stated that the tubes in which zoster convalescent sera were used in the presence of the two herpes antigens cleared more slowly than the other controls. As a rule those tubes in which specific fixation does not occur show considerable clearing after 15 minutes at 37°C., and this is complete in 20 minutes, 10 minutes before the test is read. The tubes in question took the full half-hour to clear. This did not occur where zoster convalescent serum was used in the presence of a vaccinia antigen, or in the tubes containing zoster vesicle fluid and antiherpes serum. The question arises as to whether this slowness was due to the presence of a slight degree of fixing power possessed by the human zoster convalescent sera for herpes virus. Herpes

infection in man is widespread, and it is interesting to note that one of us (S. P. B.—unpublished work) has found that normal human serum quite frequently possesses neutralizing power for herpes virus in low dilutions. A similar observation has been made by Zinsser and Tang (1929).

TABLE X.

Serum.	Antigens. Herpes guinea-pig pad emulsion, 1/20. 2 M.H.D.	Vaccinia guinea-pig pad emulsion, 1/20. 2 M.H.D.	Zoster V.F. "S." 1/15. 2 M.H.D.	Saline. 2 M.H.D.
Antiherpes B. 862, 1/20	++++	−	−	−
	+++	−	−	−
„ B. 369, 1/10	++++	−	−	−
	+++	−	−	−
Zoster convalescent, "X"	−	−	++++	−
	−	−	+++	−
„ „ "Y"	−	−	++++	−
	−	−	++++	−
Saline	−	−	−	−
	−	−	−	−

COMPLEMENT-FIXATION WITH VACCINIA VIRUS.

It has already been mentioned that some experiments were done with hyperimmune rabbit sera prepared against rabbit strains of neurovaccine and epidermal vaccinia. These experiments were unsatisfactory, and did no more than indicate that a specific reaction was involved, the specificity of which might be proved if the right technique could be found. The search for this technique was tedious and unsuccessful, and it was abandoned when it was found that such excellent results could be obtained in the case of herpes by using guinea-pigs.

Accordingly four sera were prepared by hyper-immunizing guinea-pigs against tissue suspensions of a guinea-pig strain of vaccinia (sera V. 22, V. 23, V. 24, V. 25). These suspensions were all contaminated by staphylococci and a small Gram-negative bacillus, the presence of which made it necessary to prepare a serum against them to be used as a control in the tests. For this purpose agar slopes were sown from each of the vaccinial suspensions, and after 18 hours' incubation at 37°C. the growth was washed off with 10 c.c. of saline and a guinea-pig immunized therewith. The dose employed was 1·0 c.c., the number given being the same as in the four animals receiving vaccinia virus. The concentration of bacteria was much greater in this suspension than in the vaccinial suspensions. Since the dose inoculated was 1·0 c.c. in both cases this control guinea-pig received a greater mass of bacteria (serum V. 26).

Experiments with these sera were entirely successful. The four antivaccinial sera all fixed complement in the presence of vaccinial antigens, but never did so when herpetic normal or bacterial antigens were used, and the only one (V. 25) that was so tested fixed complement equally well with an antigen prepared from rabbit vaccinia as with a suspension of infected guinea-pig pads. Three experiments are given which substantiate these assertions:

EXPERIMENT *I, 27.—The Four Antivaccinial Sera and Control Serum against Vaccinial, Herpetic and Bacterial Antigens.*

The vaccinial and herpetic antigens were prepared as usual. The bacterial antigen was a thin suspension made from the contaminants grown from the vaccinial antigen. This was heated at 56°C. for 1½ hours before use.

Serum.	Dilution.	Saline control + complement.		Antigens. Herpetic + complement.		Bacterial + complement.		Vaccinia + complement.	
		1 M.H.D.	2 M.H.D.	2 M.H.D.	4 M.H.D	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.
V. 22	1/5	−	−	−	−	−	−	++++	+++
		−	−	−	−	−	−	+++	++
V. 23	1/10	−	−	−	−	−	−	++	+
		−	−	−	−	−	−	+	−
V. 24	1/10	−	−	−	−	−	−	++++	+++
		−	−	−	−	−	−	+++	++
V. 25	1/20	−	−	−	−	−	−	++++	++++
		−	−	−	−	−	−	++++	+++
V. 26	1/5	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−
Saline controls		−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−

EXPERIMENT *I, 26.—Antivaccinial Serum V. 25 and Control Serum V. 26 against Guinea-pig and Rabbit Vaccinial Antigens and a Herpetic Antigen.*

Guinea-pig vaccinial and herpetic antigens prepared as usual. The rabbit vaccinial antigen was a 5% suspension of vesicle epithelium that had been kept frozen three weeks. The sera were used 1/20. No weakening of reaction after 24 hours in refrigerator and therefore only one reading given.

Serum.	Saline control + complement.		Antigens. Herpetic + complement.		Vaccinial (guinea-pig) + complement.		Vaccinial (rabbit) + complement.	
	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.
V. 25	−	−	−	−	++++	++++	++++	++++
V. 26	−	−	−	−	−	−	−	−
Saline controls	−	−	−	−	−	−	−	−

EXPERIMENT *I, 29.—The Four Antivaccinial Sera and Control Serum against Vaccinial Antigen and Normal Antigen containing Bacteria.*

The vaccinial antigen was made as usual. The bacterial contaminants grown from the vaccinial antigen were added to the normal antigen prepared from normal pads in a concentration of 630 million per c.c.

Serum.	Dilution.	Saline control + complement.		Antigens. Herpetic + complement.		Normal + complement.		Vaccinia + complement.	
		2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.
V. 22	1/5	–	–	–	–	–	–	++++	++++
		–	–	–	–	–	–	++++	+++
V. 23	1/5	–	–	–	–	–	–	++++	+++
		–	–	–	–	–	–	+++	+
V. 24	1/10	–	–	–	–	–	–	++++	++++
		–	–	–	–	–	–	++++	++++
V. 25	1/20	–	–	–	–	–	–	++++	++++
		–	–	–	–	–	–	++++	++++
V. 26	1/5	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–
Saline controls		–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–

CONCLUSIONS.

1. Specific complement-fixation with the viruses of vaccinia and herpes can be obtained with hyperimmune sera prepared in the guinea-pig.

2. A prolonged period of fixation is necessary for the satisfactory demonstration of this reaction.

3. Evidence has been obtained that bacterial contamination of the virus antigens plays no part in this reaction.

4. Some evidence has been produced to show that zoster vesicle fluid gives specific fixation in the presence of zoster convalescent sera.

5. The evidence adduced is further proof that virus and antibody unite outside the animal body.

We wish to express our gratitude to our clinical colleagues, Dr. W. J. O'Donovan, Dr. R. T. Brain, Dr. W. R. Brain, Dr. L. Hartston and Dr. W. W. Woods, who have been good enough to supply us with material used in this investigation.

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